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Paracoccidioidomycosis: an Update

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INTRODUCTION

Paracoccidioidomycosis is a systemic disorder that primarily involves the lungs and then disseminates to other organs and systems. Secondary lesions appear frequently in the mucous membranes, lymph nodes, skin, and adrenals. Both the clinical presentation of the mycosis and the course of the disease vary from patient to patient. Besides overt disease, subclinical infections have been documented in healthy residents of areas where the disease is endemic. These areas are confined solely to certain countries in Latin America. Paracoccidioidomycosis is frequently diagnosed in Brazil, Venezuela, Colombia, Ecuador, and Argentina; in Brazil, it constitutes an important health problem. The etiologic agent is a dimorphic fungus, *Paracoccidioides brasiliensis*, whose natural habitat is presently unknown (113, 166, 245, 312).

ETIOLOGIC AGENT

Macroscopic and Microscopic Characteristics

P. brasiliensis grows as a yeast form in cultures at 37°C and in host tissues; at lower temperatures, the fungus grows as a mold (157). In the yeast phase, the colonies are soft, wrinkled, and cream colored; growth becomes apparent after 10 to 15 days of incubation. The colonies are composed of yeast cells of different sizes (4 to 30 μm), usually oval to elongated, and have a thick refractile cell wall and a cytoplasm that contains prominent lipid droplets (157). The most characteristic feature of the yeast form is the pilot's wheel appearance, i.e., multiple budding mother cells surrounded by various peripheral daughter cells, on which cultural and histological diagnoses are based (8). Cells with single buds or short chains of blastoconidia can also be observed (157). The mold phase grows slowly (20 to 30 days at 20 to 26°C), producing small, irregular, white to tan colonies covered by short aerial mycelia which often adhere to the agar, breaking its surface. A diffuse brown pigment is produced by some isolates (157). Microscopically, the hyphae are thin and septate, and in the usual laboratory media sparse chlamydospores are the only additional structures seen (115).

Conidia. When certain isolates are cultured under conditions of nutritional deprivation, they give rise to various types of propagules, among them bulging arthroconidia and pedunculated and single-celled conidia (41, 259). When isolated from the parent mycelium (268), these propagules exhibit thermal dimorphism and give rise to either mycelial mats or multiple budding yeast cells (Fig. 1) at 26 and 37°C, respectively (259, 272). Furthermore, when given to mice by the inhalant route, conidia are infectious and the yeast cells formed from conidia produce a chronic progressive disease (182). They are also able to induce pulmonary fibrosis in mice (274) like that observed in humans.

Conidia are uninucleated, but when incubated at 37°C their transformation into yeasts results in multinucleated cells (185). According to San-Blas (286), these uninucleate propagules are produced from mycelial growth only under the stress created by adverse environmental conditions such as lack of water and nutrients.

Scanning and electron microscopy studies of the conidia have revealed finer structural details; for instance, their formation appears to be a terminal event, as most conidium-bearing hyphae collapse but have healthy looking conidia. Their small size (3.5 to 5.0 μm) is compatible with alveolar deposition. A variety of conidial types is produced (intercalary arthroconidia; septate, pedunculate conidia; and terminal hyphal conidia) (93, 285). These propagules are shown in Fig. 2 and 3. The typical subcellular organelles of physiolog-

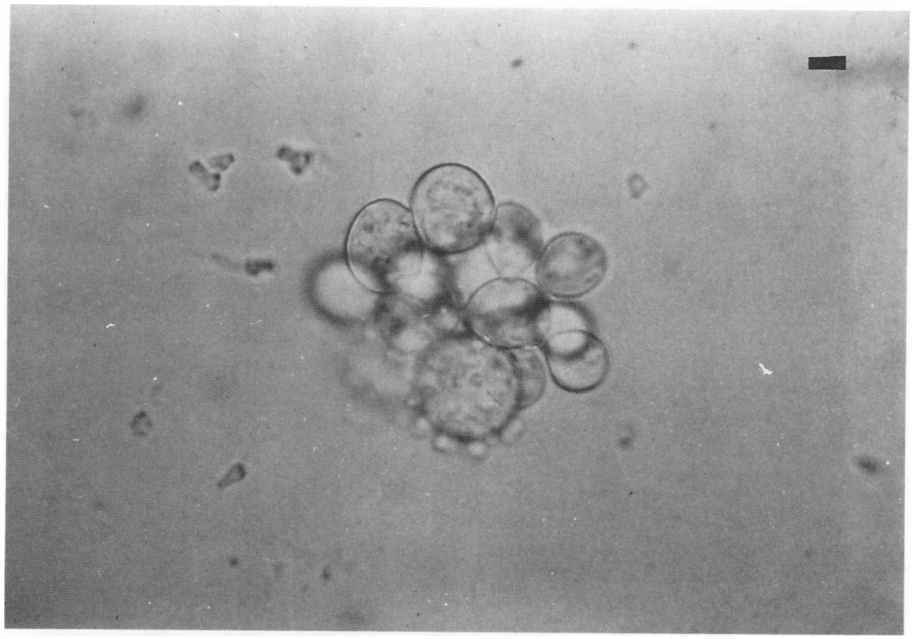

FIG. 1. Microculture of conidia incubated at 36°C. Note the size differences between untransformed conidia and those that have already converted into multiple budding yeast cells. Bar, 10 μm.

ically competent eukaryotic cells were found in the conidia by transmission electron microscopy; in mature cells, the cytoplasm appeared densely packed with food reserves such as large lipid bodies. There is a thick spore wall surrounded by a coat of delicate microfibrils (93). These findings indicate that the propagules are well equipped to survive environmental stress.

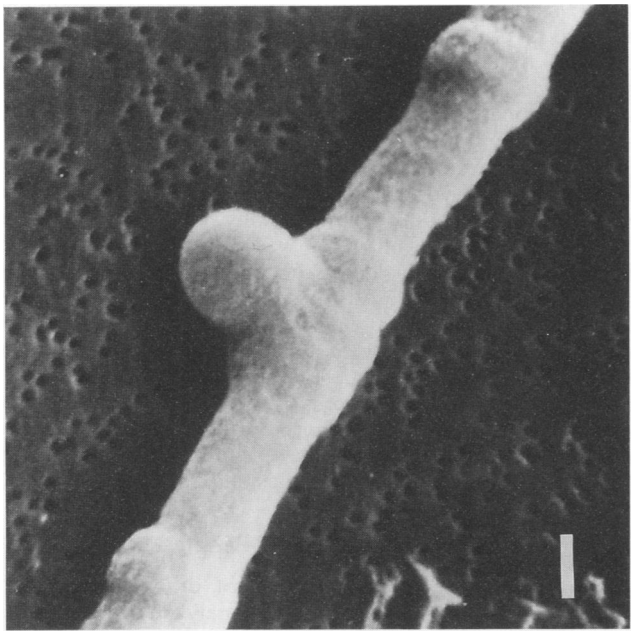

FIG. 2. Scanning electron microscopy of *P. brasiliensis* conidia; early stage in the formation of an intercalary arthroconidium. Note that the bulging occurs between adjoining septae. Bar, 1 μm. (Original micrograph prepared by W. A. Samsonoff and M. R. Edwards, Wadsworth Center for Laboratories and Research, Albany, N.Y.)

Yeast cells. Previous histochemical studies have revealed that *P. brasiliensis* yeasts are very active metabolically and that various enzymes (phosphatases, esterases, dehydrogenases, and triphosphatases) are produced during fungal growth (52).

Studies with 10 different *P. brasiliensis* isolates in their yeast forms were carried out by Kashino et al. (149, 150). They found similar ultrastructural details in all isolates. The cell wall layer was thin but presented two electron-dense sublayers. The plasma membrane was composed of three layers and presented multiple invaginations that constituted vesicles and tubular structures. Also present were large numbers of mitochondria, a scanty endoplasmic reticulum, numerous vacuoles, and multiple nuclei. Growth curves for the yeast cells were similar for the 10 isolates studied, although the mean generation time varied from 21.2 to 102.6 h. Morphology was distinctive in most of the cultures, although some irregularities were observed in two isolates that showed incomplete conversion to the yeast form. Sizes of the individual yeast cells also varied. No correlation could be found between growth curves (generation times) and pathogenicity in mice (149, 150).

Chlamydospores. Another propagule of the fungus that has received recent attention is the chlamydospore (115), a resistant cell that under adverse environmental conditions (low levels of nutrients or low oxygen supply or both) is produced in abundance. Upon appropriate incubation, it can also reproduce the parent structure. Microscopic study of such chlamydospores has revealed multiple nuclei and numerous mitochondria, indicating a capacity for further independent development (115).

Transformation. By microscopic study of the sequential steps occurring during the mycelium-to-yeast transforma-

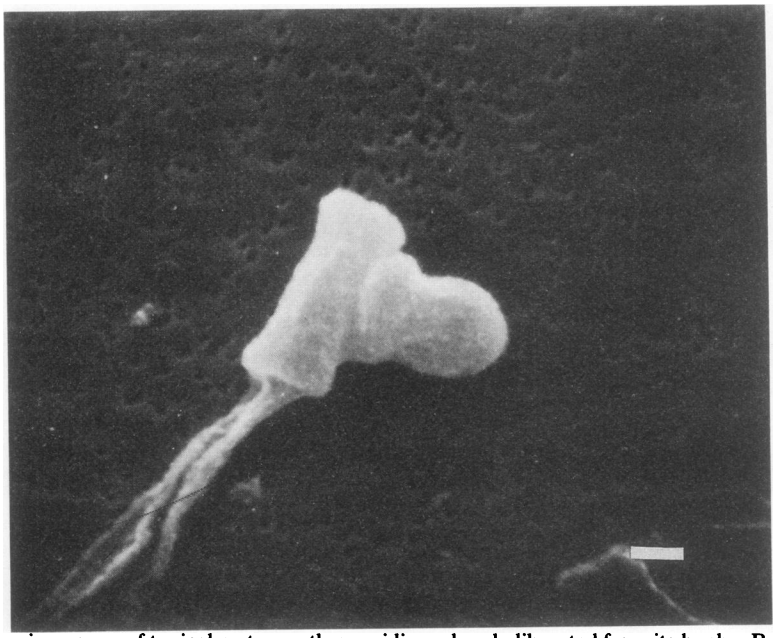

FIG. 3. Scanning electron microscopy of typical mature arthroconidium already liberated from its hypha. Bar, 1 μm. (Original micrograph prepared by W. A. Samsonoff and M. R. Edwards, Wadsworth Center for Laboratories and Research, Albany, N.Y.)

tion, the first change is the formation of intercalary or lateral swellings that resemble chlamydospores; these increase in size, acquire a double contour, and later on produce multiple buds (283).

To further explore the influence of temperature on transformation, mycelial fragments were incubated at temperatures of 23 to 37°C. Transformation began at 28°C with the chlamydospore-like structures noted above (225). A sizable proportion of mycelial elements transformed at 34°C, but multiple budding became more noticeable at 37°C (225).

Cytochemical and structural studies of the yeast form and the mycelial cell walls of the fungus were done by Paris et al. (224). The presence of beta-1,3-glucan in the yeasts, alpha-1,3-, and 1,6-glucan in the mycelium, and chitin in both forms was confirmed (292). An attempt was made to correlate the morphologic changes during dimorphism with the chemical composition of the walls, but this effort revealed that dimorphism depends not only on the presence of particular polysaccharides in the cell wall but also on their relative quantities and spatial arrangement (224). These findings confirm previous studies by Venezuelan investigators (reviewed by San-Blas et al. in reference 292).

Recently, San-Blas et al. reviewed some pertinent aspects of *P. brasiliensis* dimorphism with emphasis on the fact that transformation requires a strict control of glucan synthesis since, in vivo, alpha-glucan is the main polysaccharide within the yeast wall and there are only traces of beta-glucan; contrariwise, the latter polymer is the only glucan present in the mycelial wall (84, 287, 292). Experiments with membrane preparations of both forms of the fungus revealed that the sugar nucleotide donor in a reaction that produced glucan as the main product was UDP-glucose (287, 289). This synthetase system was partially inhibited by nucleotides, especially in the mycelial form (287, 292). Certain cytoplasmic factors, such as an acidic proteinase, can regulate beta-glucan synthesis and act as activators of beta-glucan synthetase. These effects take place at 23°C, that is, at a temperature compatible with mycelial (beta-glucan) growth (287, 292). These findings lend support to the earlier hypothesis of San-Blas and San-Blas on the dimorphism of the fungus (288).

On the same subject, the changes induced in the mycelium by increased temperature result in both the uncoupling of oxidative phosphorylation and a decrease in ATP levels which is accomplished by lowering the respiratory functions and the electron transport mechanism. These changes are followed by cessation of respiration and protein synthesis; after some time in the presence of cystine and coincident with the appearance of the yeast morphology, cytochromes accumulate and respiratory function is restored (187).

Conidia inoculated onto culture medium and incubated at 26°C grew as two distinct types of colonies, the usual mycelial colony and a yeastlike colony, called yeast at room temperature (322). The latter was indistinguishable, both macro- and microscopically, from the usual yeast phase cultures produced at 37°C. When cultures were kept at room temperature and special conditions (such as media enriched with fetal calf serum) were furnished, the yeast at room temperature aspect was preserved; otherwise, there was a quick reversion to the mycelial form (322). This variant of the fungus proved to be more virulent than the parent mycelial and yeast form cultures, indicating a relationship between phenotypic characteristics and pathogenicity (321).

Virulence and Influence of Certain Factors

Virulence was also the subject of other studies. The stability of the virulence marker was analyzed in reference to the culture history of the isolate and the methods used for its storage (37). When a murine model was used, recently isolated cultures were more virulent than old ones, indicating that subculturing leads to loss of virulence; furthermore, animal passage results in restored virulence (37). This finding should be taken into consideration when experimental ani-

mal studies are planned. Kashino et al. (151) also observed loss of virulence in one of their isolates after repeated subculturing, but the isolate recovered its original virulence by animal passage. Also, isolates (yeast form) able to grow in a microaerobic atmosphere were more virulent than those that failed to do so (295).

The in vitro actions of various disinfectants on the yeast form of the fungus were explored; formaldehyde, ethyl alcohol, and sodium hypochlorite used at appropriate concentrations are all effective in inactivating the fungus (300).

Structural changes occurred in *P. brasiliensis* yeast and mycelial cells when they were in contact with the triazole derivative itraconazole; at certain concentrations (0.07 ng/ml), the drug prevents reproduction of the yeast cells and induces necrosis of the mycelial cells (33). Amphotericin B causes marked alterations in the lipid composition of yeast cells, resulting in decreased amounts of total lipids, steroids, and certain fatty acids (135).

Oxygen and nutritional requirements. The yeast and mycelial phases of *P. brasiliensis* both require a generous supply of oxygen for growth (258); however, young yeast cells can adapt gradually to reduced oxygen tension and enter a resting stage. Resting cells can regain metabolic activity when placed under adequate conditions of oxygenation (258). This capacity may well explain the prolonged latency of this mycosis (4).

The fact that pathological samples (such as sputum, tissues, or exudates) obtained from patients with paracoccidioidomycosis and kept in the refrigerator (4°C) for teaching purposes show rapid production of mycelial elements led Bedout et al. (21) to study the survival capacity of the mycelial form in distilled water at low temperatures. They found that development of the fungus did indeed take place under these harsh conditions, but a long time (5 months) was required for an increase in fungal mass. Electron microscopic studies of the mycelium grown in water at 4°C revealed that the inter-hypha-hypha phenomenon (viable hypha growing into dead hyphal cells) took place frequently, suggesting that the fungus utilizes debris from degenerated fungal partners to fuel its continued growth (21).

The influence of iron on the growth of *P. brasiliensis* has been explored (11). Both mycelial and yeast forms can grow under conditions of iron deprivation; however, when an iron chelant was added to the culture medium, there was a delay in the initiation of yeast growth and almost total inhibition of mycelial development. In the presence of the chelant but with iron excess in the medium, the inhibitory effect was reversed, partially for the mycelium and completely for the yeast. The iron-binding capacities of the culture supernatants were at their peak in those culture media that had the lowest iron concentrations, suggesting that both fungal forms synthesize a siderophore (11). Plating efficiency has been shown to improve when *P. brasiliensis*-produced siderophores are added to culture media (66).

As shown by Paris et al. (223), the mycelial phase of the fungus appears to be prototrophic: nine isolates of the fungus were studied and found capable of growth at 23°C in the absence of essential amino acids. On the other hand, in their yeast phase the same isolates were shown to require an accessory growth factor for development, a sulfur-containing amino acid (223). However, not all researchers agree with these findings (59, 287).

The effects of different sources of nitrogen were also explored but found not to be as limiting a condition as was the use of amino acids. Growth rates, and to some extent morphology, differed depending on nitrogen source (59, 223).

Effect of hormones. One aspect of the host-parasite relationship that has proven revealing is the effect of mammalian hormones on the mycelium-to-yeast or conidium-to-yeast transformation. On epidemiological grounds alone, it is clear that female sex hormones are important in the progression of paracoccidioidal infection towards overt disease. In vitro experiments designed to determine the effects of various mammalian hormones revealed that beta-estradiol specifically inhibits the transformation of the mycelium into the yeast form (269). Further experiments demonstrated that other *P. brasiliensis* propagules, e.g., conidia, could not transform into yeast cells in the presence of beta-estradiol (284). These facts, plus the finding of an estrogen-binding protein in the cytosols of *P. brasiliensis* yeast and mycelial cells (172, 311), suggested that endogenous host estrogens act through the fungal binding protein to inhibit the transformation of mycelial and conidial propagules into the yeast form, thus delaying (or inhibiting) the adaptation of the fungus to host tissues (172, 269, 284, 311).

Studies were undertaken to determine the production of cytosolic proteins during the mycelium-to-yeast transition of *P. brasiliensis* (71). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analyses showed numerous differences between the mycelial and yeast forms. The addition of beta-estradiol to mycelial cultures before the temperature was raised to 36°C to induce transformation to yeast cells resulted in alterations in the mycelial band patterns and detection of five novel protein bands. Other changes, such as blocked synthesis of fungal proteins needed for transformation, were also recorded. These results suggest that, analogous to mammalian steroid receptor action, the functional responses of *P. brasiliensis* to estradiol are related to protein expression, which presumably is mediated via a specific binding protein-ligand complex (71–73, 172, 311). The overall results of these hormonal studies suggest that the host hormonal milieu influences fungal behavior and has important pathogenic consequences.

A radiometric technique was used to promptly measure *P. brasiliensis* growth and metabolic activity so that the effects of certain compounds on fungal development could be determined more rapidly (44). For instance, it was shown that the addition of amphotericin B (10^5 μg/ml) and diethylstilbestrol (5 μg/ml) inhibited the normal metabolism of the fungus.

The effect of rat castration on the outcome of experimental paracoccidioidomycosis induced by yeast cells was investigated (152). Females turned out to be quite susceptible: they sustained more deaths and severer pulmonary disease. Castration, which should have lowered hormonal levels, reduced the rate of infection in females and increased it in males. McEwen et al. also found that females infected with conidia had severer lung infection than males (182). According to Sano et al. (294), it is not possible to ascertain the differences in female animals infected with the yeast form unless their estrous cycles are known. Experiments along these lines revealed that, histopathologically, females (especially those at metestrus II) were more susceptible to *P. brasiliensis* yeast cells than males of the same lineage. On the other hand, females at proestrus or diestrus had the same susceptibilities as male animals. Concerning sex differences in experimental animals, it must be stressed that, with one exception (182), the inoculum used in different experiments was composed of yeast cells and yeast cells are not susceptible to hormonal influences in vitro (269). In the experi-

ments by McEwen et al. (182), a large conidial inoculum was used; some of the conidia may have escaped the inhibitory action of estradiol. It is evident that research on this topic is needed.

ECOLOGY

It is generally accepted that the habitat of *P. brasiliensis* is exogenous to humans, but the precise ecological niche remains undiscovered (243). The fungus has been isolated from soils twice (5, 213), but many similar attempts have failed (243).

A puzzling finding was that of Ferreira et al. (102), who isolated *P. brasiliensis* from a commercial dog chow. The dog being fed the chow showed signs of intoxication. The chow had been in direct contact with the soil of a farm located in an area of high humidity in Brazil. Attempts to isolate the fungus from soil samples around the farm did not succeed.

That the fungus habitat is unpredictable was shown by the findings of Gezuele (120), who isolated a species of *Paracoccidioides* from penguin excreta in Antarctica. Studies are under way to determine whether this isolate is *P. brasiliensis*. These last two isolations emphasize the problem of locating the natural microniche of *P. brasiliensis*.

The ecological characteristics of areas of endemicity and laboratory data suggest that the fungous microniche must lie in a humid environment (243). Based on the fact that most patients in Uruguay are woodcutters in indigenous forests, it has been postulated that *P. brasiliensis* resides in aquatic heterothermic freshwater animals (fish, amphibia, and others), which serve as its reservoir (74). Aquatic birds feeding on these reservoirs disseminate the fungus in nature by means of regurgitation and excretion. When their excreta later fall around trees, nests, standing water, and the like, the yeasts convert into mycelia and sporulate if given the required conditions. Humans acquire the infection by inhalation of the spores during agricultural pursuits. This hypothesis and others (243) have not yet verified the specific fungus-environment relationship.

In areas where paracoccidioidomycosis is endemic, skin testing with specific fungal antigens has not been revealing. Even though many surveys have been conducted (157, 231), results have not delimited sites of high endemicity where the fungus might be sought (243). Furthermore, there have been no reports of epidemic outbreaks which could furnish clues to a common site of exposure (243). In the past, *P. brasiliensis* infection in animals had not been demonstrated; recently, however, armadillos (*Dasypus novemcinctus*) were shown to harbor the fungus in their internal organs. Naiff et al. (209, 211) have repeatedly isolated *P. brasiliensis* from the spleens, livers, and lungs of a series of healthy armadillos collected in Para, Brazil. This animal species may provide a clue to the presence of the fungus in natural sites.

EPIDEMIOLOGY

Geographic Distribution

One of the most outstanding characteristics of paracoccidioidomycosis is its geographic distribution (i.e., restricted to Latin America from Mexico [23°N] to Argentina [34°S]); however, the disease does not occur in every country within these limits. Brazil accounts for 80% of reported cases; Colombia and Venezuela are next (243). Except for a case in Trinidad (142), the mycosis has not been reported in the Caribbean islands, the Guyanas, Surinam, or Chile. In Central America, paracoccidioidomycosis has been reported in all countries except Belize and Nicaragua. In countries where the disease is endemic, cases are not distributed homogeneously around the territory but tend to concentrate around humid forests (subtropical or tropical). Thus far, few cases have been reported from prairies, coastal regions, desert zones, and equatorial jungles. The conditions predominating in countries with a high endemicity are mild temperatures (17 to 24°C), adequate rainfall (900 to 1,810 mm/year), abundant forests, plenty of watercourses and indigenous trees, short winters, and rainy summers (243).

TABLE 1. Nonautochthonous cases of paracoccidioidomycosis reported outside areas of endemicity[a]

Country of report	No. of cases	Time (yr) between visit to endemic area and overt disease[b]		Lesions appeared when patient was in:	
		Mean	Range	Endemic area	Home country
United States	15	16.0	3–60	3[c]	11[c]
Europe	26	13.5	0.5–37	8	17
Asia and Middle East	2	16.5	3–20	2	

[a] Data taken from references 4, 131, 154, and 219.
[b] More than one country was visited by various patients.
[c] Data not available for some patients.

Forty-three cases have been reported outside the areas of endemicity (Table 1). In every case, however, the patient had visited or lived previously in one of the recognized countries of endemicity (4, 131, 154, 219). In some cases, the diagnosis of the mycosis was made while the patient was living in Latin America but he returned to his home country, outside of an area of endemicity, family or to visit to obtain medical care or both. The countries mentioned most often in the patients' medical records were Venezuela (18 cases) and Brazil (20 cases) (4, 131, 154, 219).

It is interesting that, in some of these cases, the latency period between departure from the region of endemicity and the time when overt manifestations of the mycosis became apparent was prolonged (mean, 15.3 years). This observation indicates that *P. brasiliensis* remains dormant for extended periods.

Areas of Endemicity

The long latency period so characteristic of paracoccidioidomycosis hinders the precise determination of the site where the infection was acquired (31), a fact that led Borelli to postulate, many years ago, the concept of reservarea (28). A reservarea is defined as the site where all of the factors conducive to infection exist, e.g., where the fungus has its natural habitat and where humans acquire the primary infection. These areas are molded and limited by the ecosystem (altitude, temperature, rainfall, type of soil, and type of vegetation). Areas of endemicity, defined as those places where the mycosis is diagnosed or reported or both, may or may not coincide with the reservarea, because the patient may have been diagnosed in a place different from that in which he acquired the primary infection (4).

Demographics

Age. Paracoccidioidomycosis is observed only exceptionally in children (3%) and young adults (10%) but is regularly diagnosed in adults aged 30 to 60 years (157). Skin testing with paracoccidioidin in healthy individuals in areas of endemicity indicates that, although reactivity is usually low in children and young adults, contact with *P. brasiliensis* is still important during the first 2 decades of life (131, 168, 178, 210, 231).

Sex. The mycosis occurs more frequently in males than in females, with an overall ratio in areas of endemicity of 13:1. This ratio is much larger (150:1) in Colombia, Ecuador, and Argentina (29). However, paracoccidioidin skin tests in healthy individuals from the same areas do not reveal sex differences; this indicates that both sexes acquire subclinical infections but that progression toward disease is much more frequent in males (131). Furthermore, there are no sex differences in children with overt disease (131, 165, 168, 178). These data, which indicate more frequent disease progression in males, led to the conjecture that hormonal influences are important in paracoccidioidomycosis and prompted the in vitro experimental studies described earlier. Taken together, these results tend to explain the low incidence of paracoccidioidomycosis in adult females. If at the time of the initial infection the female host has adequate levels of estradiol, the infectious propagules are unable to transform into the yeast form in tissue. Such inhibition would, in turn, allow for the expression of the specific immune defenses which, ultimately, would destroy the infecting particles (269, 284).

Occupation. Most (70%) patients are agriculturalists (29, 157); however, some cases occur in individuals who have infrequent contact with soils or vegetable matter.

Controversy still exists concerning the relationship between the increased number of male patients and the agricultural jobs they hold; however, in areas of endemicity, women are also involved in field work (131, 178).

Race. The influence of race is difficult to assess because of the frequent interracial marriages among the populations within areas of endemicity (131); whites appear to predominate in certain series of infections (157, 168, 178), and pure Indians seem to be afflicted only rarely (320). Immigrants to areas of endemicity tend to develop severe forms of the mycosis (4, 29, 131, 157). Studies of the histocompatibility antigens present in patients with this disorder have not been conclusive. Two recent reports indicate that, in comparison with matched controls, the human leukocyte antigen B40 (HLA-B40) is found significantly more often in patients from Brazil. The relative risk of developing overt paracoccidioidomycosis was 4.3 to 29.2 times higher in individuals carrying this genetic marker (130, 160). However, a previous study of Colombian patients showed that different antigens (HLA-A9 and HLA-B13) predominated; the risk of occurrence of the mycosis was 5.5 times greater among people with HLA-9 and HLA-B13 than among people with other human leukocyte antigen types. This difference could be due to the different ethnic backgrounds of the populations studied (273). In a study of 69 patients and matched controls, Demessias et al. (89) found that major histocompatibility complex class III products, especially C4B-00, are associated with chronic paracoccidioidomycosis, a circumstance that may influence the course of infection.

Contagiousness, Incidence, and Prevalence

Paracoccidioidomycosis is not contagious from person to person. Incidence and prevalence of the mycosis are difficult to determine, because notification of its occurrence is not compulsory. A report from Rio de Janeiro, Brazil, indicates that 14.6% of 500 adults hospitalized in various local centers, including a chest hospital, had paracoccidioidomycosis (106). About five new cases with overt disease occur per million patients per year (29, 131). However, the estimated number of infected individuals in the entire area of endemicity, where approximately 90 million people currently live, is close to 10 million (29, 131). Paracoccidioidomycosis is indeed important and may become more so with the exploitation of indigenous forests and increased travel and migration (29).

PATHOGENESIS

The pathogenesis of paracoccidioidomycosis has not been precisely defined, mainly because of a lack of knowledge concerning the habitat of the etiologic agent, *P. brasiliensis* (243). However, on the bases of both animal experimentation and clinical data, the possible sequence of events can be surmised. When experimental animals are infected with conidia by the inhalant route, these small (approximately 4 μm) propagules reach the distal portions of the lungs, where they transform into yeast cells and grow in the lung parenchyma, producing a progressive disease that disseminates to extrapulmonary organs (182). It is likely that humans also acquire the infection in this fashion.

In a competent host, fungal growth is halted and the interaction ends with no apparent damage to the host (subclinical infection). In such a setting, the primary foci disappear and the fungus is usually destroyed, but host cells retain a "memory" of the infection. If the host-parasite balance is upset by immunosuppression or other causes, then the infection progresses and gives rise to full-blown disease (123, 198).

Clinical Forms

Two forms of the disease are distinguished: the acute (subacute) juvenile form and the chronic adult form. The former runs a more rapid course and is severer than the latter (116, 123, 198, 222). In both cases, however, cell-mediated immune functions are abnormal, and in the absence of specific therapy, mortality is high (91, 157, 168). When specific therapy is given, improvement can be expected; nonetheless, lesions usually remain as sequelae (residual form). If such lesions harbor viable *P. brasiliensis* cells, relapses may occur (113, 116, 198). Remission is often accompanied by significant pulmonary fibrosis (55, 132, 212).

The disease may arise either directly from a primary focus with no latency period or, more commonly, by reactivation of quiescent foci (endogenous reinfection). Exogenous reinfection leading to symptomatic disease is also possible (113, 123, 198). Once established, the mycosis adopts one of two clinical forms, infection or disease.

Infection

There is evidence of the existence of subclinical infection in paracoccidioidomycosis. Thus, *P. brasiliensis* has been found in residual, partially calcified lesions in individuals being evaluated for conditions other than the mycosis (7).

Development of the pulmonary primary complex with lymphatic involvement and satellite adenopathy in a patient with lung carcinoma has also been documented (298). Furthermore, subclinical infection occurs rather frequently in healthy residents of areas of endemicity as shown by skin reactivity to paracoccidioidin (231).

Disease

Juvenile form, acute or subacute. The juvenile form represents only 3 to 5% of all cases. This form is characterized by a rapid course (weeks to months) and by marked involvement of the reticuloendothelial system (spleen, liver, lymph nodes, and bone marrow). Cell-mediated immune function is severely depressed in these patients, most of whom are children or young adults. This is the severest form and the one with the worst prognosis (165, 317). The clinical picture is characterized by reticuloendothelial system organ hypertrophy and bone marrow dysfunction and is often mistaken for a lymphoproliferative disorder or, if severe dissemination has occurred, for a septicemic episode (165, 317). The involved mesenteric lymph nodes may undergo hypertrophy, leading to bowel obstruction and/or an acute abdominal syndrome (165, 317). Often biopsies show large numbers of actively multiplying *P. brasiliensis* cells with no granuloma formation (114). In this particular form of the disease, the lungs are seldom the primary focus as there are no special clinical or radiologic manifestations (165). Even so, a search for the fungus in pulmonary secretions is usually positive, indicating that the lungs are also involved (271). The radiologic pattern is variable, with hypertrophied hilar lymph nodes and infiltrates, mostly basal, predominating (165, 317).

Chronic form, adult type. The adult form occurs in more than 90% of patients, most of whom are adult males. The disease progresses slowly and may take months or even years to become fully established. Unlike the symptoms of the juvenile type of disease, pulmonary manifestations are evident in 90% of adults (113, 132, 163, 168, 169). In approximately 25% of cases, the lungs (rarely other organs) are the only system clinically afflicted (unifocal). However, in some cases, the unifocal pulmonary involvement may be silent and the patient seeks medical advice only after dissemination has led to extrapulmonary lesions (multifocal form) (113, 163, 168, 265, 266).

Respiratory symptoms are nonspecific and include cough, expectoration, and shortness of breath; weight loss, fever, and anorexia have also been recorded. Pulmonary lesions revealed by X rays are nodular, infiltrative, fibrotic, or cavitary; they are often bilateral and preferentially located in the central and lower portions of the lungs, with the apices remaining free of disease (55, 163, 168, 226, 265). Respiratory signs and symptoms are often minor and do not correspond to the extensive lung involvement frequently revealed by X rays (8, 55, 80, 168, 170, 265).

There is a resemblance to tuberculosis, with which the mycosis coexists in 10% of cases (8, 168). However, the radiological presentation may also suggest neoplasia, idiopathic interstitial fibrosis, and other disease entities (8, 55, 163, 168, 265). Respiratory function tests reveal that lung damage is mostly of the obstructive type, and in severe cases ventilatory insufficiency leads to cor pulmonale (8, 55).

In the chronic multifocal form, the symptoms are variable and referred to more than one organ or system. Most frequently, lesions occur in the oral and nasal mucosa, skin, lymph nodes, and adrenal glands (1, 8, 113, 163, 168, 265, 316). Table 2 presents the frequency of organic involvement according to three series of cases published by different investigators (56, 168, 212).

TABLE 2. Main organic involvement in a series of 352 patients with paracoccidioidomycosis

Organ(s) involved	No. of affected patients studied by:			Total no. (%)
	Campos et al.[a]	Naranjo et al.[b]	Londero and Ramos[c]	
Lungs	42	43	185	270 (76.7)
Mucous membranes	24	26	172	222 (63.0)
Skin	19	16	6	41 (11.6)
Lymph nodes	16	23	8	47 (13.3)
Spleen, liver	15	2	2	19 (5.4)
Adrenals	0	9	0	9 (2.5)
Others	2	2	3	7 (2.0)

[a] Reference 56; 47 patients.
[b] Reference 212; 45 patients.
[c] Reference 168; 260 patients.

Less frequently, patients present with ocular involvement (83, 305), central nervous system disease (117, 318), bone destruction, vascular system pathology, and even genital lesions (8, 54). Scans making use of gallium-67 frequently reveal lesions that were not suspected clinically or radiologically (42, 122).

In this mycosis, thyroid impairment, although rare, has been detected by autopsy. To investigate thyroid function, 25 patients with the disease and 20 matched controls were studied. Levels of T_4 (thyroxine) and T_3 (tri-iodothyronine) and the patient's responses to thyrotropin-releasing hormone were measured, but no sign of altered thyroid function was detected (153).

Depending on the patient's general condition and immune status, the chronic form of the mycosis can be mild, moderate, or severe (111). Usually, humoral immunity is preserved, and the patient exhibits polyclonal activation of B cells (68). Cell-mediated immune functions are depressed in the severe, multifocal form of disease but preserved in less serious cases. The tissues tend to show granuloma formation (111).

Residual. Usually, and regardless of the organ involved, paracoccidioidomycosis heals by fibrosis. The corresponding sequelae may permanently interfere with the well-being of the patient (55, 56). The most incapacitating and frequent residual lesions occur in the lungs, where various associated abnormalities, including cor pulmonale, may appear (8, 55, 132, 170). Dyspnea and cardiopulmonary restriction are observed in 60 to 80% of patients. Dysphonia, microstomy, and stenosis of the glottis and trachea with associated dysphonia have also been recorded. Infection of the adrenal glands may seriously impair gland function (8, 55, 114, 168).

Most adult patients respond to specific therapy, especially to the imidazole derivatives (215, 255). However, the fibrotic sequelae persist despite therapy that is adequate to arrest the disease process (212, 255).

LABORATORY DIAGNOSIS

The definitive diagnosis of paracoccidioidomycosis can be accomplished only by laboratory procedures. The development of mycological techniques for the diagnosis of paracoccidioidomycosis (156, 232, 240) has paralleled progress in methods of detecting antigens and antibodies (97, 214, 242,

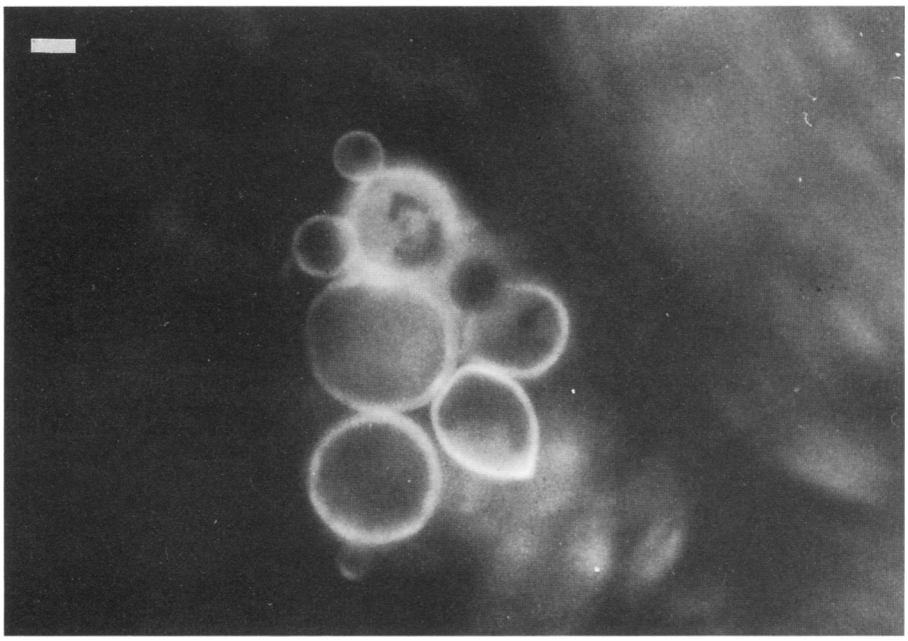

FIG. 4. Calcofluor white fresh preparation from a pathological specimen showing a *P. brasiliensis* round mother cell surrounded by spherical to elongated daughter cells. Note smaller secondary conidia in one of the daughter cells as well as a thick cell wall. Bar, 10 μm.

279, 306). Detection of delayed-type hypersensitivity (DTH) also assists in diagnosis (96, 98, 217). Relevant test systems will be presented in this section.

Microbiology

Specimens. Sputum, bronchoalveolar lavage fluid, crusts, material from the granulomatous bases or the outer edge of ulcers, pus from draining lymph nodes, cerebrospinal fluid, or tissue biopsy samples are all adequate for the mycological procedures described here. These procedures involve visualization, isolation, and identification of the microorganism (156, 163, 174, 195, 221, 232, 240).

Microscopy. The best and speediest way to establish the diagnosis of this fungal disorder is by direct examination of clinical specimens, which allows detection of the fungal elements. A number of procedures and stains (KOH, calcofluor, and immunofluorescence) can be used for this purpose (134, 148, 156, 195, 232). In a clinical specimen, *P. brasiliensis* appears as globose yeast cells with multiple buds. Younger cells measure 2 to 10 μm in diameter, and mature cells measure ≥30 μm. Some mother cells may reach 60 μm in diameter. The cells have a thick refractile wall (0.2 to 1 μm); are spherical, oval, or elliptical; and may occur in chains of four or more (Fig. 4). From one to a dozen narrow-necked buds of uniform or variable sizes may arise from the mother cells. Sometimes, the yeast cells appear in chains and have single buds, elongated distorted cells, and a number of other forms (195, 240). Cooper (78) described a case of pseudoparacoccidioidomycosis in which the yeast phase of *Mucor circinelloides* bore a superficial resemblance to that of *P. brasiliensis*.

Histopathological examination with hematoxylin and eosin, methenamine silver, Papanicolaou, and periodic acid-Schiff stains (114, 155, 240) and indirect immunofluorescence (IIF) procedures (148, 240) of infected tissues reveal a pyogranulomatous process with infiltrating polymorphonuclear leukocytes (PMN), mononuclear cells, macrophages, and multinucleated giant cells. Multibudding yeast elements are the characteristic structure, and finding them establishes the diagnosis (113, 114, 319). Occasionally, however, exceedingly small, pseudohyphal, and hyphal forms of *P. brasiliensis* are seen in tissue sections (155, 171, 240).

Sputum samples are one of the most important specimens used for diagnosis; accordingly, several procedures to improve the detection of organisms in sputum have been developed (156). Recently, a cell block preparation method in which smears are stained with methenamine silver has proved to be more sensitive than simple direct examination (181). In a series of studies by different groups in Latin America, the sensitivity of the direct examination (wet mounts, smears, and histopathology) varied from 85 to 100% (12, 56, 106, 167, 169, 265, 266) (Table 3).

Accurate quantitation of viable fungal populations to be used for in vitro studies has been achieved by use of the following dyes: methylene blue, erythrosin B, Janus green,

TABLE 3. Results of microbiological tests in the diagnosis of paracoccidioidomycosis

No. of patients	% of positive samples examined by:		Reference
	Microscopy	Culture	
39	95	86	266
41	100	ND[a]	167
19	85	100	265
34	100	ND	169
47	100	ND	56
18	89	ND	12
56	88	ND	106
Total of 254	93.8	93.0	

[a] ND, not done.

acridine orange (126), and fluorescein diacetate-ethidium bromide (249). In one experimental study, the first three stain techniques were unreliable and the acridine orange estimates were difficult to reproduce (125). Janus green has been used regularly with good results by Kashino et al. (150, 151). Fluorescein diacetate-ethidium bromide has been claimed to be the best indicator of viability; its results are reliable and reproducible (249). Lately, this vital dye has been used in several experimental designs (2, 37, 38, 63, 66, 220, 300).

Culture. In 1908, Lutz (174) was the first to isolate *P. brasiliensis* in culture. He used Sabouraud agar incubated at room temperature and described the growth as resembling "pelos de ratinhos brancos" (white mouse hair) (174). The primary isolation of fungi causing pulmonary diseases is not always successful because sputum, the clinical sample most frequently examined, has abundant contaminating resident flora (232). The addition of antibacterial agents and mold inhibitors to isolation media has resulted in improved recovery rates (156, 157, 232, 252). Nevertheless, it is advisable to culture repeated samples in a battery of selective and nonselective media. The use of digestion and concentration procedures for mucous samples has been recommended as a means of achieving higher isolation rates (156, 240). The effects of culture media and digestion procedures on the recovery of fungal pathogens, including *P. brasiliensis*, were explored by Restrepo and Cano (248), who found that modified Sabouraud and yeast extract agars were the best isolation media. Treatment with mucolytic reagents did not increase recovery rates but was useful for producing a homogeneous product (248).

The mycelium-to-yeast or yeast-to-mycelium transition in *P. brasiliensis* depends largely on the temperature of incubation. In the laboratory, the recovery of the fungus from pathological samples is regularly made on artificial media incubated at room temperature (approximately 25°C). Growth is slow (20 to 30 days), and a variety of colonial forms can be obtained. Microscopic observations of the mycelium show only thin septate hyphae (3 to 4 μm in diameter) and intercalary chlamydospores (15 to 30 μm). Because the mycelial form is not distinctive, dimorphism must be demonstrated by subculture at 37°C. At this temperature, *P. brasiliensis* grows rapidly and produces a cream-colored colony. Under the microscope, smears prepared from this culture exhibit oval to spherical cells 4 to 30 μm in diameter, some of which show one or several round to oval daughter cells emerging from the periphery. The large yeast cell (mother cell) bearing multiple buds (pilot wheel) is specific to this fungus (156, 157, 240).

The isolation rates of the fungus in culture in two studies were 86 to 100% (265, 266) (Table 3). In 1964, Pedroso found that semianaerobic conditions in synthetic media were an important factor in the isolation of *P. brasiliensis* (228). More recently, it has been shown that the yeast cells of this fungus can assume a resting stage under microaerophilic incubation (253, 258); similar conditions may exist in closed human lesions. Such a resting phase may explain the prolonged survival of the fungus in lesions (7).

A rapid and specific method for the immunological identification of *P. brasiliensis* mycelial form cultures, which sporulate sparsely, was introduced in 1980 (309). The extraction of specific cell-free soluble antigens (exoantigens) allows detection of specific precipitin bands by immunodiffusion (309).

Viability studies based on the isolation of *P. brasiliensis* in culture have been conducted with the idea of determining plating efficiency. Generally, this efficiency is expressed as the number of CFU divided by the hemocytometer count of viable units. When standard mycological media are used, the results are poor (126, 249). Data obtained with a synthetic medium (257) supplemented with culture filtrates (spent medium) and horse serum markedly improved the plating efficiency (66), and this medium has been used in several experimental designs (37, 63). *P. brasiliensis*-produced siderophores were likely to be the growth-enhancing moiety in culture filtrates. A later experiment showed that the yeast form of *P. brasiliensis* produced higher levels of iron chelants in media containing low concentrations of iron (11). The production of germ tubes in a slide culture is also a dependable procedure for determining the viability of *P. brasiliensis* yeast form cells (2, 127, 249).

The maintenance of cultures of *P. brasiliensis* over long periods resulted in changes in fungal cell walls and decreased virulence (37, 63, 151), but virulence could be recovered by culturing the isolate in complex culture medium or by passing the isolate in vivo (37, 63, 293). The method introduced by Castellani (66a) for maintaining fungal cultures in water was shown to be useful for *P. brasiliensis* mycelial forms; cultures remained viable for 8 months and the microorganism actually increased in mass under these conditions (21). The viability of *P. brasiliensis* after liquid nitrogen cryopreservation has also been demonstrated (88), and this procedure is widely used to maintain some collections.

Gene probes. Recently, assays with DNA probes based on the detection of specific rRNA sequences have been developed for the rapid identification of cultures suspected of being *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Coccidioides immitis*, and *Cryptococcus neoformans* (70). Identification is possible with a sensitivity of 100% for the target organisms. With the exception of the *B. dermatitidis* probe, which also responded to *P. brasiliensis*, the specificity of the test was 100%. Negative results were obtained with all other nontargeted organisms tested (70). Currently, DNA probes for *P. brasiliensis* are not available.

Immunodiagnosis

Immunoserology. The first report on the immunodiagnosis of paracoccidioidomycosis appeared as early as 1916. In that year, Moses studied the complement fixation (CF) reaction, his being one of the first studies on the immunodiagnosis of deep mycoses (201). Since that time, many studies on the use of serologic methods in paracoccidioidomycosis have been published; they are reviewed in depth by Fava-Netto (97, 98), Negroni (214, 216), and Restrepo (242).

Emphasis has been placed on the most suitable methods for the preparation of antigens in order to enhance sensitivity, specificity, and reproducibility of tests (26, 49, 99, 100, 239, 324). A variety of test systems have been employed for immunodiagnosis; some of them determine nonspecific indicators of infection (C-reactive protein) (214). Most tests, e.g., tube precipitation, agar gel immunodiffusion, counterimmunoelectrophoresis, or immunoelectrophoresis (77, 97, 260, 323), measure soluble reactants. Some tests, e.g., latex agglutination (263), erythroimmunoassay (47), and immunofluorescence (112, 261), measure antigen-antibody reactions. Lytic assays (CF) (97) and an enzyme-linked immunosorbent assay (ELISA) have also been described (45, 57, 193). Data taken from many different studies make it evident that different tests do not always give similar results for the same serum. Consequently, it is advisable to employ more than one test for clinical diagnosis (242).

TABLE 4. Immunoglobulin classes as specific anti-*P. brasiliensis* antibodies

Assay	% of patients with:			Reference
	IgG	IgM	IgA	
IIF	ND[a]	68	ND	203
IIF	100	33	61	16
IIF	98	45	33	24
ELISA	100 (acute)	100	100	191
		47 (chronic)		

[a] ND, not done.

In general, patients with paracoccidioidomycosis are not deficient in specific antibody production. About 90% of patients with clinical disease have specific antibodies at the time of diagnosis. Furthermore, in disseminated disease, antibody production is elevated and antibody titers are high (146, 241, 244). Thus, serological tests are of value for both the diagnosis and the prognosis of paracoccidioidomycosis (241, 242). Levels of immunoglobulin classes have been determined by several groups. When a patient is first diagnosed and for the first year thereafter, elevated immunoglobulin G (IgG) levels are common; IgM levels, on the other hand, tend to be normal (10, 16, 24, 68, 79, 264, 278, 304). IgA levels may be increased, normal (10, 16, 79, 264), or decreased (304). An elevation of IgE was found in some patients with active paracoccidioidomycosis who manifested a depression of cellular immunity (10, 328). However, since specific IgE antibody constitutes only 0.6% of total IgE, other explanations besides a relationship between T-cell dysfunction and overproduction of IgE must be considered. Further observations confirmed the presence of high levels of IgE in untreated patients; these levels decrease with treatment, and positive correlations between the number of B cells and the IgE levels and between the IgE levels and depressed leukocyte migration inhibition in assays with *P. brasiliensis* antigen have been observed (190).

An increased polyclonal immunoglobulin response was observed by Robles in 15.3% of patients, most of whom presented with severe disease (278). The polyclonal B-cell activation response was the subject of a later study (68); the results showed that the number of IgG-secreting cells was significantly elevated in paracoccidioidomycosis patients. Increased IgG and IgA levels in serum and more circulating immune complexes were also recorded in these patients (69).

There have been efforts to correlate the class of anti-*P. brasiliensis* immunoglobulins detected in patient sera with the clinical form or the stage of the disease process (Table 4). IgM antibodies were detected for the first time by Mota and Franco (203); even though IgM antibodies were present in 68% of patients with all types of disease, there was no correlation between elevated levels of IgM in serum and a given clinical presentation. Furthermore, IgM antibodies did not correspond to precipitating antibodies (203) (Table 4). In an animal model, the role of the IgM antibody was defined. Levels of mouse IgM antibodies rose, peaked, and decreased fairly early, whereas levels of IgG antibodies rose, peaked to high titers, and remained high thereafter (64). Corresponding findings in humans remain to be determined but would have epidemiological implications.

In other investigations (16), no constant association between a given form of the disease and the presence of IgG (100%), IgM (33%), or IgA (61%) anti-*P. brasiliensis* antibodies was found (Table 4). Nonetheless, it was noted that IgM antibodies occurred frequently in patients with lymph node lesions, while IgA antibodies prevailed in patients with illnesses of <1-year duration (16). Measurement of total IgG, IgM, and IgA anti-*P. brasiliensis* antibody levels by IIF revealed that anti-*P. brasiliensis* IgG, IgM, and IgA could be detected in 98, 45, and 33% of cases, respectively (24) (Table 4). There was also a tendency toward higher levels of anti-*P. brasiliensis* IgG among patients with the acute progressive form of disease (83.4%). On the other hand, IgG was detected less frequently in patients with chronic, more localized forms, both multifocal (68%) and unifocal (55.5%) (24). Recently, Mendes-Giannini et al. (191) found that the sera of patients with paracoccidioidomycosis contained IgG-, IgA-, and IgM-specific antibodies to a 43-kDa antigen of *P. brasiliensis*. IgG and IgA were present in all patients observed (acute or chronic form of disease). Concerning the IgM response, patients with the acute form had 100% reactivity, whereas only 47% of patients with the chronic form were reactive. A lowering of IgG, IgA, and IgM antibody titers also correlated with clinical improvement (191).

There appears to be no constant relationship between the levels of various classes of immunoglobulins in serum and their specific antibody functions against *P. brasiliensis* (241). Sometimes, the concentration of IgG parallels the activities of complement-fixing or -precipitating antibodies detected by gel diffusion (79, 214, 278). A study to determine the nature of the precipitating antibodies concluded that they are of the IgG class (108). The correlation between the level of anti-*P. brasiliensis* IgG and a positive tube precipitin test suggested that precipitin-type antibodies are also of the IgG class (24).

Monoclonal antibodies have been prepared by two different groups. Puccia and Travassos produced antibodies against the 43-kDa glycoprotein to foster their experimental studies on its excretion and proteolysis (236). Figueroa et al. prepared monoclonal antibodies to define species-specific epitopes, and they discussed the potential use of monoclonal antibodies in serodiagnosis and the development of an ELISA for the detection of circulating antigen (107).

Circulating immune complexes in paracoccidioidomycosis patients were first described by Arango et al. (9) and later confirmed by Chequer-Bou-Habib et al. (68, 69). These observations were made in patients who exhibited depressed T-cell responses. For this reason, it was suggested that immune complexes could be involved in the genesis of the cell-mediated immune depression observed in paracoccidioidomycosis patients (3, 9, 68, 69).

The role of antibodies in paracoccidioidomycosis patients has not been determined. The evidence argues against effective protection, because in severe disease antibody production is elevated (241). However, animal studies have shown that antibodies may constitute an important mechanism of defense. When high- and low-antibody-responder mice were infected with *P. brasiliensis*, the highest mortality and the most extensive dissemination were found in the low responders (60). Genetic susceptibility cannot be excluded.

Antibodies seem to be protective, as shown by Kamegasawa et al. (147), who studied the effect of vaccination on the presentation of ocular lesions in guinea pigs. Vaccinated animals who had responded with high antibody titers had less ocular involvement than controls (28 versus 85%) (147).

Antigens. Over the years, *P. brasiliensis* antigens derived from either culture filtrates or whole yeast cells have been used successfully for diagnosis. A description of the ideal antigen as given by Negroni in 1972 (214) is as follows: easy

to prepare, long shelf life, specific (no cross-reactions), reproducible, sensitive, reliable, and reactive in more than one serological test. Different antigen preparations vary greatly in quality, depending on fungal strain, morphological phase, culture medium, size of inoculum, incubation time, and recovery techniques (242, 290). According to their origin, *P. brasiliensis* antigens have been classified as cell wall-derived, cytoplasmic (intracellular), and culture filtrate (exocellular) antigens (99, 250, 251, 270, 290).

Cell wall antigens were used in quantitative precipitation and immunodiffusion tests; the results suggested that galactomannans were the principal components of the antigenic preparation and that *P. brasiliensis* and other pathogenic fungi had some antigens in common. Therefore, the usefulness of cell wall antigens for skin and serologic testing was limited (290).

Cytoplasmic antigens were used by Moses in 1916 (201) in the first serologic study of paracoccidioidomycosis. He used somatic antigens obtained from broken *P. brasiliensis* cells and the CF test. His antigen detected antibodies in 8 of 10 patients tested (201). Fava-Netto's polysaccharide antigen, which was extracted from autoclaved yeast cells, came next. This preparation has been one of the most widely employed cytoplasmic antigens (94, 99). CF tests, tube precipitation reactions, and skin testing studies have all been conducted by several Brazilian groups with this type of preparation (242).

Recently, three new cytoplasmic yeast antigens have been prepared (61, 186, 250). They have the advantage of being reproducible, although stringent control of proteolysis is required for proper preservation (250). A yeast-derived cytoplasmic preparation was subjected to fractionation and protein characterization; two of the proteins had molecular weights of 66,000 and 85,000 (40).

Characterization of *P. brasiliensis* yeast form antigens has recently been attempted by Casotto (61). Using the Western (immunoblot) technique, Casotto found that the 48- and 45-kDa antigens were specific for paracoccidioidomycosis and demonstrated that immunoblot analysis of patient serum is a useful tool for the identification of immunogenic cellular components. In that study, *B. dermatitidis* had a protein pattern very similar to that of *P. brasiliensis* and the largest number of *P. brasiliensis* antigen recognition sites. The same immunoblot technique was used to characterize cellular yeast extracts of three different *P. brasiliensis* isolates (62). There was antigenic variability not only between the isolates but also between antigens with the same molecular masses. Similar results have been obtained by different investigators with these and other isolates (23, 297).

Antigens released into the culture medium during microbial growth (yeast or mycelial form) are frequently used in the serodiagnosis of paracoccidioidomycosis. These antigens contain all kinds of soluble cellular products: cell wall polysaccharides such as galactomannans, cytoplasmic proteins, and/or glycoproteins. Two reviews on the topic have been published by Yarzábal (324) and San-Blas and San-Blas (290). Restrepo and Drouhet (254) made the initial attempts to characterize the antigenic moieties that are present in culture filtrate preparations and that are responsible for reactivity in the serological tests. When a culture filtrate prepared from the yeast phase of *P. brasiliensis* was analyzed by electrophoresis, five antigenic fractions (labeled A through E) were regularly observed; arc A was detected in every serum sample with precipitating activity. A similar culture filtrate antigen and selected positive control sera were tested in an immunodiffusion test by Restrepo and Moncada, who identified three precipitation lines, designated 1, 2, and 3 (262). A similarity between band 1 and precipitin arc A was proposed (262).

Another attempt to characterize metabolic antigens from the mycelium and yeast phases of *P. brasiliensis* was made by Yarzábal et al. (51, 323, 326). Two components with cathodic migration, E1 and E2, were separated by immunoelectrophoresis. E1 had alkaline phosphatase activity and appeared responsible for the early appearance of precipitins in infected or immunized animals (325); E2 was considered the specific diagnostic antigen (307, 327).

During the last 10 years, the Brazilian group led by Travassos has been searching for a specific antigen (48–50, 235, 237, 308, 314). Their findings point toward a 43,000-molecular-weight glycoprotein (gp43) as the specific antigenic component in *P. brasiliensis* culture filtrates. This protein can be isolated in pure form by gel filtration column chromatography or Sepharose-patient IgG affinity chromatography. Immunoprecipitation of ^{131}I-labeled gp43 and immunodiffusion tests were positive with paracoccidioidomycosis patient sera and with hyperimmune rabbit serum produced against the band E2 antigen of Yarzábal (235). Further experiments, which labeled *P. brasiliensis* with ^{35}S-methionine, showed that gp43 is continuously produced and excreted in the medium by yeast cells in the exponential phase of growth (308).

In an attempt to standardize the production of a serodiagnostic antigen, Camargo et al. (49) monitored the growth curve of the yeast form of *P. brasiliensis*, searching for the excretion of the 43-kDa antigen. In immunodiffusion tests, they established that a 7-day crude exoantigen displayed a sensitivity and specificity similar to those of the purified gp43 antigen. Their results also demonstrated the reproducibility and long-lasting stability of the product when it was kept lyophilized (49). Attempts to increase the yield of gp43 by using a liquid culture medium enriched with tomato juice (TOM medium) were successful (236). Clones expressing epitopes of the gp43 diagnostic antigen have also been described by the same group (314). When an immunoblotting technique was used with paracoccidioidomycosis sera, the 43-kDa glycoprotein was recognized by 100% of the sera and a 70-kDa glycoprotein was recognized by 96%. Those authors concluded that antibodies to both glycoproteins can be considered markers for human paracoccidioidomycosis (50).

In a recent study, the deglycosylated form of the 43-kDa glycoprotein was compared with the native antigen in regard to antigenicity, excretion, and susceptibility to proteolysis (236). The deglycosylated product had a molecular weight of 38,000, was secreted in small amounts from the cell, was immunogenic, and appeared more susceptible to proteolysis than the native antigen (236). From these data and from two additional observations (237, 314), it was established that the peptide epitopes of the gp43 are immunodominant in their reactions with patient antibodies, rabbit hyperimmune sera, or monoclonal antibodies (235, 236). Cross-reactions detected with histoplasmosis and lobomycosis sera were attributed to carbohydrate epitopes (237).

The possible role of this exocellular antigen as a virulence factor for humans has been explored by two different groups (194, 236). It was found that gp43 by itself has a proteolytic activity at pH 5.6 but not at neutral pH. The caseinolytic and collagenolytic activities were attributed to metal-dependent proteinases (194). However, it is still unclear whether the fungal proteolytic activity operates within the host by digesting structural proteins in tissues (236).

Recently, the exoantigen test was refined by Camargo et

al. (48), who used a simple and rapid method for extracting specific cell-free antigens. The extract was obtained from a 3-day-old culture, had a protein content of 200 to 300 μg/ml, and was a reliable reagent for several serological tests (48).

Lytic assays. Two tests, CF and complement-mediated lysis, have been standardized for use in the serodiagnosis of paracoccidioidomycosis (94, 220). The initial CF studies by Moses were done in 1916 (201). In 1949, Lacaz et al. began to use CF procedures on a regular basis and demonstrated that most patients had detectable antibody titers (158). These two studies were followed by the methodical trials of Fava-Netto, who in 1955 standardized the test and demonstrated that it was very valuable for the diagnosis and follow-up of patients with the mycosis (94, 95). His experience allowed him to draw the following conclusions, which are still valid: (i) CF antibodies appear late in the course of the disease and continue to be present for long periods, sometimes even after clinical cure; (ii) CF titers are low in the mild, localized forms and high in the disseminated forms of the disease; and (iii) titers tend to rise with relapses. In the acute forms and following successful treatment, antibody titers fall rapidly, whereas in the chronic forms, they taper off slowly. The simultaneous use of more than one test was recommended (94, 98). Two recent publications dealing with the sensitivity, specificity, efficiency, and predictive values of the serological tests have reinforced the utility of the CF test in the diagnosis and follow-up of patients with paracoccidioidomycosis (58, 87).

The detection of antibodies by the complement-mediated lysis assay has recently been evaluated with the aim of amplifying the battery of serodiagnostic tests capable of demonstrating the presence of active paracoccidioidomycosis (220). Even though lytic antibodies were present in various clinical forms of the disease, no positive correlation between lytic antibody levels and precipitin titers or IIF test titers was observed (220).

Precipitation reactions. The versatility of the serological reactions that use soluble antigens has been exploited in paracoccidioidomycosis (214, 242). Tube precipitation, agar gel immunodiffusion, counterimmunoelectrophoresis, and variations of these techniques have been standardized by different groups (77, 94, 239). In Fava-Netto's experience (97), precipitins as measured by the tube precipitation technique are the first antibodies to appear during the course of the disease and the first to disappear when the patient improves with therapy. In the tube precipitation test, approximately 86% of patients with active disease prove reactive (97).

Agar gel immunodiffusion has demonstrated its versatility as both a screening test and a diagnostic test (6, 106, 164, 196, 214, 260). It is simple to perform and also highly reliable (53, 103, 105). In studies measuring the sensitivity and specificity of the immunodiffusion test, values of 89 and 91% for the former and 100% for the latter parameter have been established; as a consequence, the positive predictive value of this test is 100% (58, 87). Quantitative immunodiffusion has been used by several groups (261, 278, 303). In general, the titers are lower than those obtained with the CF test; nonetheless, the simplicity of the test is an advantage for small laboratories (261) (Table 5). Counterimmunoelectrophoresis has also been employed by several investigators (13, 53, 77, 105); its sensitivity is the same as or even slightly superior to that of immunodiffusion (87). Another variation of the precipitation test, immunoelectrophoretic procedures, has also been standardized but is used for research more than for diagnostic purposes (76, 323, 326, 329).

TABLE 5. Results of serological tests at time of diagnosis of paracoccidioidomycosis

No. of patients	% Reactivity of serological test				Reference
	CF	AGID[a]	IIF	TP[b]	
39	79	92			266
1,073	97			60	97
86	100	97			214
40	73	90			6
19	67	89			265
169	ND[c]	94			164
196	68	66	85	50	196
73	ND	100			106
Total of 1,695	81	90	85	55	

[a] AGID, agar gel immunodiffusion.
[b] TP, tube precipitation.
[c] ND, not done.

Agglutination. Only two tests that use particulate antigen carriers (latex beads and sheep erythrocytes) have been described for the serodiagnosis of paracoccidioidomycosis (47, 263). A latex agglutination test employing paracoccidioidin-sensitized latex particles was described several years ago (263). The sensitivity of the test was 61 to 69.5%, depending on the antigen bound to the latex. Cross-reactions were frequent but could be reduced by setting a titer of 32 (1:32 dilution of serum) as the minimal positive reaction (263). In the erythroimmunoassay, an antigen conjugate able to bind erythrocytes was used to quantitate antibodies to *P. brasiliensis*. Absorption with dead *Candida albicans* cells was necessary to decrease cross-reactions (47).

IIF tests. The IIF technique was introduced by Restrepo and Moncada in 1972 (261). There have been several modifications to the method by Brazilian investigators, who consider it useful for the serologic diagnosis and follow-up of patients with the mycosis (16, 24, 67, 112, 196, 203). In general, the test has the advantage of allowing the study of anticomplementary serum samples. Furthermore, a relationship between serological results and the severity of the disease has been clarified with this technique (24).

Correlations between titers given by the different quantitative tests are dissimilar (67, 112, 196), a fact that emphasizes the necessity of using more than one technique for serodiagnosis. A recent publication evaluated the reliability of some serological tests and concluded that for titers of ≥1:64, the IIF test has an efficiency of about 80%, with higher specificity than sensitivity (90 and 65%, respectively) (87).

Immunoenzymatic assays. The first immunoenzymatic assay described for paracoccidioidomycosis was performed with *P. brasiliensis* yeast cells fixed on microscope slides and used immunoperoxidase. Cross-reactions with sera from patients with histoplasmosis and candidiasis were noted (233). ELISAs with various but high sensitivities have been described (45, 46, 57, 161, 193, 237, 302); however, the different antigenic preparations employed (partially purified or crude extracts) make it very difficult to compare the results. It must be stressed that cross-reactivities have been very high (especially with sera from patients with histoplasmosis and lobomycosis) in the different systems (193, 237). Absorption of paracoccidioidomycosis sera with *H. capsulatum* yeast and mycelium components renders the test more specific, at least in some of the systems described (45, 46, 57, 191, 193, 237) (Table 6).

TABLE 6. Results of ELISAs for paracoccidioidomycosis

Antigen (concn)	No. of samples tested	% Sensitivity	Cross-reactions (%)	Positive readings	Reference
Culture filtrate (yeast) (10 μg/ml)	69	100	Histoplasmosis (45)	>1:80	193
E2 (20 μg/ml)		100	Lobomycosis (44)		193
Culture filtrate (yeast) (10 μg/ml)	20	95	Histoplasmosis (9) Candidiasis (5)	>1:400	45 45
Cytoplasmic culture (yeast), MELISA[a]	33	94	Histoplasmosis (14)	1:400	46
Cytoplasmic culture (yeast) (30 μg/ml)	101	66	Histoplasmosis (35) Coccidioidomycosis (56)	1:128	57 57
gp43 (10 μg/ml)	120	100	?	1:40	191
gp43	50	100	Histoplasmosis (53)	?	237

[a] MELISA, magnetic enzyme-linked immunosorbent assay.

Antigenemia. The description of immune complexes in patients with paracoccidioidomycosis (9, 68, 69) and the observation of precipitin lines occurring between serum samples set up in the immunodiffusion and counterimmunoelectrophoresis systems suggested for the first time that there was circulating antigen in patient sera (280). Subsequently, attempts have been made to diagnose this mycosis by detecting circulating antigens in serum samples. Several techniques with different sensitivities have been used: inverted linear rocket immunoelectrophoresis (176), immunoelectroosmophoresis-immunodiffusion (119), passive hemagglutination inhibition (177), immunoblotting (192), immunoradiometric assay (104), and ELISA (118).

More recent studies aimed at detection of the 43-kDa soluble glycoprotein (192) confirmed the diagnostic and prognostic value of antigenemia tests for acute and chronic forms of the disease. An immunoradiometric assay using the IgG fraction of rabbit antisera to *P. brasiliensis* allowed the detection of cellular and metabolic antigens at concentrations 1,000 and 100 times less than those required by the double-immunodiffusion test (104). Recently, a competitive ELISA was developed (118). This assay could detect 6 ng of antigen per ml of serum. The highest frequency of positive tests was found in patients who had the severe acute form of the disease. However, there were also false-positive reactions with sera from patients with other systemic mycoses (118).

DTH. The first recorded attempt to demonstrate DTH in patients with paracoccidioidomycosis was that of Fonseca and Area-Leao, who intradermally injected a mycelial filtrate into two patients, both of whom proved reactive (109). Since then, numerous investigators have employed exocellular and intracellular *P. brasiliensis* antigens to study skin reactivity in both infected patients and healthy populations (14, 75, 85, 92, 136, 179, 180, 210, 267, 296).

In 1961, Fava-Netto and Raphael (101) reported the use of the polysaccharide antigen (intracellular and cytoplasmic) for skin testing. This antigen was originally devised for serological tests (CF and tube precipitation). Positive reactions were obtained in 67% of proven cases of the disease, but many positive cutaneous reactions (87%) were also observed among controls (patients with other diseases and healthy controls), indicating the presence of subclinical infections (101). In the same study, depressed skin test reactivities in patients with the severe and chronic forms of the disease were pointed out. Results of some of the skin test surveys conducted to date are presented in Table 7.

At present, skin testing with a variety of antigens (paracoccidioidins) is a useful complementary diagnostic tool to evaluate the immune status of patients with various clinical manifestations of the disease (217). A reversion from nonreactive to reactive during the course of therapy indicates that cell-mediated immune responses are being restored; this signals a good prognosis (217).

CELL-MEDIATED RESPONSES

Depression of cell-mediated immune responses is a common finding in paracoccidioidomycosis and correlates strongly with the acute progressive form of the disease (188, 189, 205). It has been hypothesized that impaired cell-mediated responses in paracoccidioidomycosis are caused by the infection itself and that they contribute to the success of the pathogen. A strong argument in support of this hypothesis is that impairment of cell-mediated responses is reversible by successful therapy (197, 264). The search for a factor(s) responsible for negative immunoregulation of cellular immunity during paracoccidioidomycosis has ranged from unidentified inhibitory factors in patient plasma (208, 264) and immune complexes (9, 68, 69, 304) to an imbalance in T-cell subsets (200, 204).

Macrophages

It has been shown that monocytes and monocyte-derived macrophages support the intracellular replication of ingested *P. brasiliensis* (199). However, monocyte-derived macrophages activated by gamma interferon (IFN-γ; 300 U/ml, for 3 days) significantly (65 to 95%) inhibited the intracellular replication of ingested *P. brasiliensis* (199). These findings indicate that lymphokines (IFN) play an important role in human resistance to paracoccidioidomycosis.

PMNs

Human peripheral blood PMNs can ingest *P. brasiliensis* yeast cells and alter their stainability (127). It is still controversial whether PMN can be fungicidal for ingested yeast

TABLE 7. Summary of paracoccidioidin skin test surveys carried out in areas of endemicity[a]

Decade	No. of surveys	No. of persons tested	Country	Antigen	Positivity range (%)
1950	12	2,695	Brazil Uruguay	Culture filtrate (mycelial)	2–42
1960	9	7,341	Brazil Colombia	Polysaccharide Culture filtrate (mycelial)	4–66
1970	26	18,059	Brazil Venezuela Colombia	Polysaccharide Culture filtrate (mycelial-yeast)	6–61
1980	6	4,912	Brazil Panama Guayana	Polysaccharide Culture filtrate (mycelial) Culture filtrate (mycelial)	6–50
Total	53	33,007			5–55

[a] Modified from Lacaz et al. (157) and Pereira and Barbosa (231).

cells (36, 125). It has been shown that human PMNs could not kill an attenuated strain of *P. brasiliensis* that was readily killed by murine PMNs (36). The influence of serum on in vitro digestion of *P. brasiliensis* by human neutrophils has recently been explored (124). Results revealed that sera from patients with paracoccidioidomycosis did not inhibit the digestive capability of PMNs from healthy controls. Serum from healthy controls did not enhance digestion by patients' PMNs, cells that have been reported to be defective in destroying ingested *P. brasiliensis* yeast cells. The effect of activated or inflammatory PMNs on ingested *P. brasiliensis* remains to be determined.

Langerhans Cells

Gimenez et al. (121) examined skin biopsy samples from patients with the mycosis and found a significant reduction in the number of Langerhans cells in comparison with the number in healthy controls; furthermore, morphological alterations were noted. This suggests that a possible depression of cell-mediated immune responses might also occur at the antigen-presenting level.

Natural Killer Cells

Natural killer cells have recently been recognized in paracoccidioidomycosis patients (230). A study of 34 untreated patients revealed that all patients had an increased number of natural killer cells, which were less active than normal cells in killing a tumor target cell, K562. Consequently, their participation in resistance might be inadequate. That healthy natural killer cells might have a role in host defenses was suggested by in vitro studies with murine cells (143). Murine splenic cells with the characteristics of natural killer cells were reported to inhibit the growth of *P. brasiliensis* yeast phase targets in vitro.

Lymphocytes

Proliferative responses. Hyporeactivity of patient peripheral blood lymphocytes (PBLs) to *P. brasiliensis* antigens or mitogens has been reported by several investigators (82, 205, 208, 264). One study in which assays were done with fetal bovine serum instead of autologous serum showed no significant differences between healthy and symptomatic patient lymphocyte responses in 14 of 17 patients. In this study, symptomatic patients were not classified as to clinical forms of the disease, which in later works were found to be important (205).

An important factor in assessing the hyporeactivity of PBLs in paracoccidioidomycosis is the use of autologous serum versus healthy control serum or fetal bovine serum in assays. Responses of patient PBLs to mitogen or antigen in autologous serum were lower in most, but not all, cases than responses measured with healthy control serum (205, 208, 264). Moreover, a patient's serum had a suppressive effect on the proliferative responses of PBLs from healthy individuals (205, 208). The influence of a serum factor on proliferative responses of PBLs has also been reported in other fungal diseases caused by thermal dimorphic fungal pathogens, e.g., coccidioidomycosis (81). The suppressor activity in patient sera could be removed by absorption with protein A, implicating monomeric IgG as a suppressor factor (81). Taken together, it appears that the hyporeactivity of PBLs in terms of proliferative responses to stimuli is not necessarily an inherent lymphocyte defect but reflects the regulatory milieu in which PBLs have to respond.

T-Cell Subsets

Peripheral blood. The availability of monoclonal antibodies specific for subsets of PBLs has provided an excellent tool for determining subset profiles of PBLs in patients with infectious diseases. Attention has been focused on the ratio of $CD4^+$ (T-helper-inducer) and $CD8^+$ (T-suppressor-cytotoxic) cells because lower ratios have been associated with impairment of cellular immunity (204).

A possible relationship between depressed cellular immunity in paracoccidioidomycosis and reduced CD4/CD8 T-cell ratios has been investigated (204). In the acute form of the disease, there was a clear reduction in the CD4/CD8 ratios (mean, 1.2; range, 0.7 to 1.5; $n = 15$) compared with ratios in healthy controls (mean, 1.9; range, 1.5 to 2.4; $n = 26$). In the chronic form of the disease, the association of reduced CD4/CD8 ratios was not as clear-cut; nevertheless, 29 to 45 patients had ratios below 1.5. Similar results have recently been obtained by Tapia et al. (315) and Bava et al. (20). The latter authors also found that, when patient's mononuclear blood cells were challenged by interleukin 2 (IL-2), production of IFN was below normal. These results suggest that, in

a patient with paracoccidioidomycosis, there are alterations not only in lymphocyte subsets but also in the cell's ability to produce regulatory cytokines (20). Despite these findings, the significance of reduced CD4/CD8 ratios in systemic mycoses is difficult to evaluate in terms of cause and effect. It has been postulated that the predominance of CD8 T cells accounts for the depressed cellular immunity in patients, e.g., active suppression (111), but there are no data at this time to demonstrate this possibility.

Bronchoalveolar lavage fluid. The study of cells obtained from bronchoalveolar lavage fluid of paracoccidioidomycosis patients has been investigated by Tapia et al. (315), who found that CD4/CD8 ratios were abnormal for this type of sample. Although the proportion of T-helper-inducer cells was higher in bronchoalveolar lavage fluid than in the peripheral blood of the same patients, it was lower than in healthy persons; this finding suggests an ongoing immune response at the site of infection.

Granulomas. The study of granuloma formation relative to cellular immunity in patients with paracoccidioidomycosis has been of interest for some time (113). The association of compact localized granulomas with effective cellular immunity and healing and of loose, diffuse granulomas with impaired cellular immunity and progressive disease (113) has prompted the analysis of granulomas at the T-cell subset level. Data from such analyses provide clues to possible roles for T-cell subsets in immunoregulation.

Immunohistochemical staining techniques using monoclonal antibodies specific for CD4 and CD8 T cells revealed a predominance of CD4 T cells in granulomas from patients with the acute and chronic forms of the disease (200). CD4/CD8 ratios in granulomas were higher in biopsy samples from lymph nodes (3.8 to 4.0) and skin (1.27 to 4.27) than in those from mucosa (1.01 to 2.76). Moreover, CD4/CD8 ratios were not significantly different in compact and loose diffuse granulomas (200). It was speculated that recruitment of CD4 T cells from the blood and localization in granulomas might account for low CD4/CD8 ratio in peripheral blood. The recent subdivision of CD4 T cells into types Th-1 and Th-2 (202) and their identification in granulomas may lead to a better understanding of their roles in immunoregulation in granulomas and paracoccidioidomycosis.

Lymphokines and Cytokines

MIF. The capacity of PBLs from skin test-positive individuals to produce migration inhibitory factor (MIF) in response to specific antigen was one of the earliest in vitro correlates of active cellular immunity. The inhibition of leukocyte migration from a capillary tube was evidence of MIF production. This assay was used in studies of cellular immunity in patients with paracoccidioidomycosis (207, 304). In two studies (197, 208), when leukocytes from four skin test-negative patients were stimulated with antigen, three failed to produce MIF and migrated from the capillary tube. In contrast, leukocytes from six of six skin test-positive patients responded to antigen with MIF production and inhibition of leukocyte migration (208). At this time, MIF production and inhibition of leukocyte migration are considered advantageous for host resistance because they keep effector cells at the proper site (208). Lack of production of this and other lymphokines in paracoccidioidomycosis would understandably result in impaired cellular immunity.

TNF. Tumor necrosis factor (TNF) alpha is a cytokine produced by endotoxin-stimulated or IFN-γ-activated monocytes or macrophages. It is cytotoxic for certain tumor cells (281). TNF can also have immunoregulatory effects, for example, activation of PMNs, and act as a second signal of IFN-γ-primed macrophages (281). Detection of elevated levels of TNF in the sera of patients with paracoccidioidomycosis has been reported recently (301). Twenty-seven of 30 patients had a mean level of 300 U/ml of serum, which is three times the upper limit found in healthy control serum (301). On the other hand, Bava et al. (20) found that IL-2 stimulation of peripheral blood monocytes from adult patients with paracoccidioidomycosis resulted in less TNF being released. Whether this difference is due to methodology remains to be determined. The significance of TNF in paracoccidioidomycosis is unclear at this time.

Rezkallah-Iwasso et al. (275) found that patients with active paracoccidioidomycosis had a decreased number of T cells expressing IL-2 receptors. All of these findings indicate that lymphokine production is altered in patients with this mycosis and that such alteration is reflected by the observed depression of cell-mediated function.

OPPORTUNISM

Transplantation

Suppression of the transplant recipients' immune rejection system for organ or bone marrow transplantation has opened the door to certain opportunistic and nonopportunistic fungal infections (137). A well-documented case of an apparent reactivation of paracoccidioidomycosis in a kidney transplant patient has been reported (313). Maintenance immunosuppressive therapy with azathioprine (50 mg/day) and prednisone (10 mg/day) from 1969 to 1980 constituted the predisposing factor for development of pulmonary paracoccidioidomycosis. The patient lived outside the area of endemicity for 39 years prior to occurrence of clinical paracoccidioidomycosis in 1980. This case should alert clinicians to the possibility of reactivation of latent *P. brasiliensis* during maintenance immunosuppressive therapy. We have not found comparable reports on acquisition of primary paracoccidioidomycosis during maintenance immunosuppression after transplantation in areas of endemicity.

Cancer Chemotherapy

Hematologic and lymphatic malignancies per se, but not solid cancers, are considered to compromise host defenses. However, chemotherapeutic treatment of patients with solid tumors can predispose to reactivation of latent mycoses (27, 282). This is particularly true for patients with paracoccidioidomycosis, in whom latent infection becomes an acute pulmonary fatal disease subsequent to chemotherapy of oat cell carcinoma treated with intravenous cyclophosphamide (1 g), vincristin (1 mg), and adriamycin (50 mg) (299). Considering the results of this therapeutic regimen, i.e., severe suppression of cellular immunity, alternatives for cyclophosphamide should be considered.

There are other reports in the Latin American literature about the association of paracoccidioidomycosis and chemotherapy of cancer in areas of endemicity (175, 238). For example, there was an association of paracoccidioidomycosis and cancer patients (2.1%) in general practice cancer services in some areas of endemicity (175, 238).

TABLE 8. Characteristics of seven paracoccidioidomycosis patients with AIDS

Patient		Form of paracoccidioidomycosis	Location of lesion	First diagnosis		Outcome	Reference
Age (yr)	Sex			Mycosis	AIDS		
37	M	Subacute juvenile	Liver Spleen Bone marrow	x		Death	227
29	M	Acute juvenile	Liver Spleen Abdominal lymph nodes Inguinal lymph nodes	Simultaneous		Survival	227
43	M	Subacute juvenile	Lymph nodes Skin	x		Death	129
31	M	Acute juvenile	Lungs Skin	x		Survival	15
35	M	Subacute juvenile	Lymph nodes Lungs Skin	Simultaneous		Survival	22
?	M	Subacute juvenile	Spleen Blood	Simultaneous		?	133
29	F	Subacute juvenile	Spleen Blood	Simultaneous		Survival	128

AIDS

The spread of infection by human immunodeficiency virus (HIV) into certain areas of South America has become a serious public health problem. The recent estimated number of HIV infections in Latin America and the Caribbean equals that of North America (1,000,000) (25). The greatest incidence of HIV-positive and AIDS cases occurs in areas where *P. brasiliensis* is endemic in the population centers of southern Brazil (Rio de Janeiro and São Paulo). However, in spite of the 4,000 cases of full-blown AIDS and the 300,000 HIV-positive individuals in Brazil (129), the reported number of AIDS patients with paracoccidioidomycosis is minimal (only seven) (15, 22, 128, 129, 133, 227). An analysis of seven of the published cases (Table 8) reveals that all of these patients came from Brazil, all but one were males, and the mean patient age was 34 years. In spite of their adult age, all patients exhibited the acute or subacute juvenile form of the mycosis, a type of disease not regularly observed in patients older than 25 years. In non-AIDS patients, this form is also accompanied by a marked decrease in cell-mediated immune functions (111). All patients had multifocal involvement, with the reticuloendothelial system being involved in six of the seven. The mycosis was diagnosed before AIDS in two of the patients, after the AIDS diagnosis in another three, and simultaneously with the AIDS diagnosis in the remaining one. Recently, a brief communication reported a case of paracoccidioidomycosis in a Brazilian woman with full-blown AIDS (128).

There have been no published reports of paracoccidioidomycosis in AIDS patients in countries other than Brazil. The low incidence of paracoccidioidomycosis in AIDS patients could be explained by lack of exposure; for example, AIDS is predominantly an urban disease, whereas paracoccidioidomycosis occurs in rural areas (159). In 40 paracoccidioidomycosis patients, anti-HIV-specific antibodies were investigated, but only 1 patient gave a positive reaction. This patient was not traceable (159); further studies should be carried out along these lines. It is still not clear whether AIDS is a predisposing factor for reactivation of latent paracoccidioidomycosis, acquisition of primary disease, or neither. The data do not clarify the picture (Table 8).

EXPERIMENTAL IMMUNOLOGICAL STUDIES

Animal Models

A murine model of pulmonary disseminated paracoccidioidomycosis has been developed over the past several years (38, 63). This well-characterized experimental model has been utilized extensively for immunological studies of paracoccidioidomycosis. Hamsters (141, 229) and guinea pigs (147) have also been used for experimental studies. An array of other animals have been employed for the study of experimental paracoccidioidomycosis. An analysis of results from such studies can be found in the review by Iabuki et al. (140).

Humoral responses. In progressive disseminated paracoccidioidomycosis, humoral immune responses can be measured as serum antibody to *P. brasiliensis* antigens. Both mouse and hamster models have been used in such studies (64, 141). In mice, antibodies to antigen could be detected by ELISA as early as 3 weeks after infection (64), a time when CFU in the lungs numbered 10^4 (63). The IgM isotype response preceded the formation of IgG antibody to *P. brasiliensis* antigens (64). As the infection progressed, IgM antibody titers waned and high-titer IgG antibodies to antigen persisted. In other studies, these results were confirmed and polyclonal IgE antibodies were measured by an ELISA (138). Levels of polyclonal IgE in serum continued to rise as the disease progressed, reaching levels of 9 μg/ml of serum at 12 weeks. IgE levels fell to background levels (<0.5 μg/ml) when *P. brasiliensis* was cleared by antifungal (SCH 42427) therapy. These humoral responses in mice with murine paracoccidioidomycosis resemble those reported in humans with paracoccidioidomycosis, i.e., high titers of serum antibody to antigen (264) and polyclonal IgE (328).

Cellular responses. Cellular immune responses were measured during murine paracoccidioidomycosis. Cutaneous DTH reactions to antigen were assayed by the footpad method. Good DTH responses were made after the first week of infection, indicating a vigorous initial cellular response (64). However, DTH reactions to antigen decreased to nonsignificant levels by 8 weeks, a time when IgG antibody titers peaked and disease was severe.

Proliferative responses of peripheral blood mononuclear cells to antigen or the mitogen concanavalin A were determined by the [^{3}H]thymidine uptake method. Proliferative responses to antigen early (2 to 6 weeks) after infection became insignificant by 12 weeks. Mitogenic responses to concanavalin A became impaired by 16 to 18 weeks after infection (20% of normal) (64). These cellular response profiles for murine paracoccidioidomycosis parallel those reported for human paracoccidioidomycosis (197, 208).

Dialyzable leukocyte extracts obtained from healthy or *P. brasiliensis*-immunized hamsters (141) were used to transfer cell-mediated immunity (229). The recipients of the extracts from immunized animals presented high levels of cellular immunity to the fungus, as detected by MIF and a histologically confirmed DTH response. Such an immune response lasted for 4 months. The possibility of using a similar, more refined dialyzable leukocyte extract for immunotherapy is presently being investigated (229).

That both cellular immunity and hormonal responses are important in host defense was demonstrated by Calich et al. (43). Using susceptible (B10A) and resistant (A/SN) mouse strains, they demonstrated that the former has depressed DTH responses to *P. brasiliensis* and altered humoral responses as well as macrophage dysfunction. Resistant animals, on the other hand, had no such abnormalities (43).

In vitro studies with murine peritoneal and pulmonary macrophages revealed that yeast form *P. brasiliensis* is readily phagocytosed by macrophages (Fig. 5) and that yeast cells replicate intracellularly in normal macrophages (35). When macrophages were activated by lymphokines or recombinant IFN-γ, they were able to kill 50% of ingested yeast cells in 4 h and sterilize cultures in 24 to 48 h (35). Pulmonary macrophages from mice given 4×10^5 U of IFN intraperitoneally 24 h earlier had significantly enhanced abilities to kill *P. brasiliensis* in vitro (34); moreover, transmission electron microscopic studies showed that killing of *P. brasiliensis* by activated macrophages was evidenced by disruption of mitochondrial integrity and then plasma membrane damage and cytoplasmic disintegration. The cell wall remained intact and did not show signs of attack (39).

A role for PMNs in murine resistance to *P. brasiliensis* is not clear at this time. It has been reported that peripheral blood PMNs or immunologically elicited (activated) PMNs could kill 40 to 70% of attenuated but not virulent strains of *P. brasiliensis* (36, 183).

Immunoregulation. Modulation of cellular responses in vivo and in vitro has been demonstrated in the murine model of paracoccidioidomycosis. Mouse serum with high titers of antibody to *P. brasiliensis* (0.5 ml given intravenously 1 day before testing) significantly depressed DTH to antigen in reactive mice (65). Peripheral blood mononuclear cells from mice with overt paracoccidioidomycosis (18 weeks after infection), when mixed with normal peripheral blood mononuclear cells (1:1), depressed normal peripheral blood mononuclear cell proliferative responses to concanavalin A by 74 to 95% (65). T-cell depletion experiments that removed Lyt-2.2 cells from the PBL of infected mice (65) enabled the remaining cells to respond normally to concanavalin A. In other work in which immune responses to antigen in healthy mice were studied, evidence was obtained for induction of T-suppressor cells by antigen alone (144, 145). More recent studies with anti-IL-4 antibody indicate that neutralization of IL-4 during the first weeks of infection abrogate enhanced polyclonal IgE production and reduce disease progression (138). These findings merit further investigations to clarify immunoregulation in paracoccidioidomycosis.

ANTIFUNGAL AGENTS

In Vitro Susceptibility Testing

Broth dilution method. MICs of antifungal agents for clinical isolates of *P. brasiliensis* have been determined by a broth dilution method with the endpoint read visually as no growth after 5 to 7 days of incubation with shaking at 35°C. Yeast form cells of *P. brasiliensis* are grown at 36°C in modified McVeigh-Morton medium (257). A suspension of 10^5 cells per ml in broth is used as the inoculum. Twofold dilutions of an antifungal agent, e.g., sulfamethoxazole or trimethoprim (310), starting at 2 mg/ml are added to a series of inoculum-containing tubes. Synergistic activity of two agents can be assessed with a checkerboard array of inoculum-containing tubes. In one study, three of four patient isolates were resistant at >200 μg of trimethoprim per ml. On the other hand, a combination of drugs in a ratio of 5:1 of sulfonamide-trimethoprim was effective and synergistic. The MIC for the most susceptible isolate was 0.97 μg of sulfamethoxazole per ml and 0.20 μg of trimethoprim per ml, and the MIC for the most resistant isolate was 46.9 μg of sulfamethoxazole per ml and 9.4 μg of trimethoprim per ml.

MICs of azoles, e.g., ketoconazole, itraconazole, and fluconazole, for *P. brasiliensis* isolates can also be determined by this method. MICs of azoles alone have been reported to be approximately 0.1 μg/ml, and those of amphotericin B are 0.5 μg/ml (162).

Efficacies of New Azoles in a Murine Model

It is known that the in vitro antifungal activity of an agent may not coincide with the in vivo efficacy during disease. Consequently, it is important to show the efficacies of antifungal agents in animal models of the diseases prior to clinical trials.

Acute murine paracoccidioidomycosis. A murine model of acute pulmonary paracoccidioidomycosis in young (4-week-old) BALB/c male mice (38, 184) has proven useful for testing the relative efficacies of experimental antifungal agents. An oral itraconazole regimen, even at 10 mg/kg/day, was shown to be protective (Fig. 6). Nonfatal infections can also be used to evaluate relative efficacies of imidazoles (ketoconazole and BAY/9139), triazoles (BAY/7133 and ICI 153,066), and polyenes (amphotericin B) (162).

Chronic progressive paracoccidioidomycosis. A murine model of chronic pulmonary and disseminated paracoccidioidomycoses (38, 63) has been used to test the efficacies of antifungal agents in treating established disease. In this model, treatment was withheld until 1 month after infection. Oral (gavaged) ketoconazole (100 mg/kg twice a day) was effective after 4 weeks in clearing disseminated *P. brasiliensis* from the spleen and, when continued for 8 weeks, in significantly reducing the number of CFU of *P. brasiliensis* in the lungs (139).

THERAPY OF HUMAN PARCOCCIDIOIDOMYCOSIS

Sulfonamides

Until 1940, paracoccidioidomycosis was considered an incurable disorder; in that year, Ribeiro (276) introduced sulfonamides (sulfamidopyridine) and found them to be helpful in the treatment of this disorder. Subsequently other sulfonamide derivatives were employed, particularly sulfadiazine, which is effective in 70% of patients and is still used

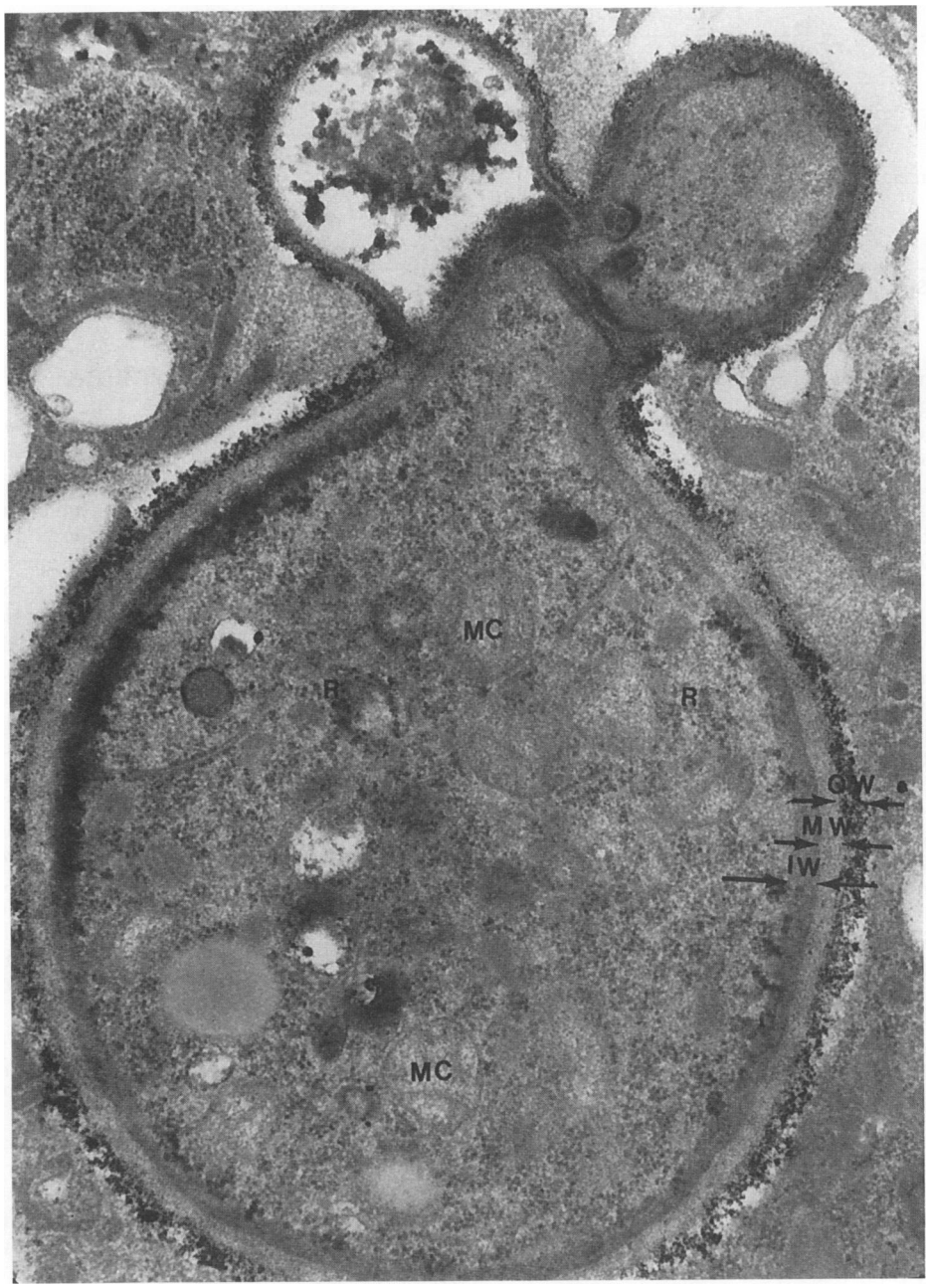

FIG. 5. Ultrastructure of *P. brasiliensis* 4 h after ingestion by nonactivated macrophages. Magnification, ×37,800. OW, outer wall; MW, middle wall; IW, inner wall; MC, mitochondria; R, ribosomes. (From reference 39, with permission.)

(86). Sulfonamides have several advantages (low cost and relatively low toxicity), as well as shortcomings such as the long periods of treatment required (up to 5 years) and the significant rate of relapse (25%) (32, 86). Relapse is often accompanied by induced resistance of the fungus (247). Although the overall response rate to sulfonamides is gratifying, the long-term results are less than satisfactory, as an important proportion of the patients (approximately 30%) will relapse or die within 10 years of discontinuation of therapy (32). The use of the trimethoprim-sulfonamide combination has been recommended as an alternative for patients with sulfadiazine-resistant isolates (173). Recently, attempts have been made to improve the efficacy of sulfadiazine by combining it with trimethoprim (cotrimazine) (17); preliminary results indicate that such a combination may be useful for the treatment of cerebral paracoccidioidomycosis, as both drugs penetrate the cerebrospinal blood barrier well (17).

The levels of sulfonamide in serum must often be measured to determine the dose and the frequency with which these compounds are to be administered. The levels, however, are affected by renal function and by genetic factors linked to liver metabolism (acetylator phenotype). With these considerations in mind, a study was undertaken in a

TABLE 9. Current therapies for paracoccidioidomycosis

Medication	Daily dose (mg)	Recommended length of therapy	Result at end of treatment (%)			Relapse (%)
			Remission	Death	Worsening	
Sulfonamide						
Sulfadiazine	6 g	3–5 yr	69	21	9	35
Sulfamethoxypyridazine	500					
Amphotericin B[a]	0.5–0.75	3–4 mo	47	26	27	38
Ketoconazole	200	6–12 mo	92	0.4	6.6	11
Itraconazole	100	3–6 mo	95	0	0	3.5

[a] Dose per treatment. Sulfonamide therapy should follow amphotericin B treatment.

group of patients treated with cotrimazine to explore the relationships among their renal function, acetylator phenotype, and levels of sulfadiazine in serum (19). Approximately 95% of the patients had adequate sulfadiazine levels, i.e., >40 μg/ml. In the remaining cases, lower concentrations were found; these cases failed therapy. The highest levels of free sulfadiazine were found in patients characterized as slow acetylators (19).

Amphotericin B

The next effective drug, introduced in 1958, was amphotericin B (158). This polyene proved to be more effective than sulfonamides and has since become a major tool for the treatment of patients with severe disseminated disease (56, 91, 246). Administered as indicated for other deep-seated mycoses and even in smaller, well-regulated doses (30), this drug achieves remission of most symptoms in 50 to 60% of patients, many of whom are in poor condition at diagnosis. Unluckily, its known side effects and difficulties of administration are deterrents to its use; furthermore, even when combined with sulfonamides, the relapse rate with amphotericin B is still quite high (20 to 30%) (32, 91). The efficacy of amphotericin B also depends on physician supervision, which is not always available in areas where the disease is endemic (32). It has been customary to follow the course of amphotericin B with prolonged oral sulfonamide therapy to maintain remission (32, 86, 91).

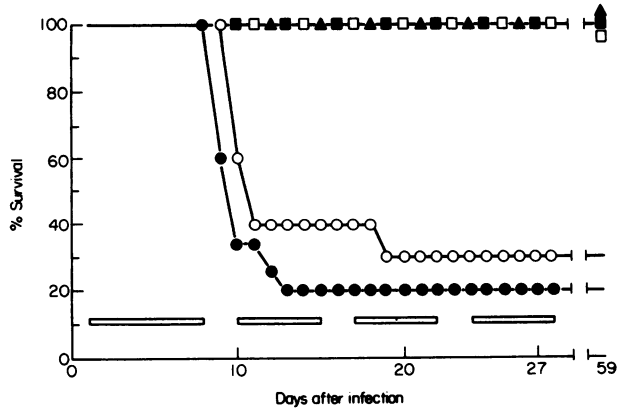

FIG. 6. Survival following pulmonary challenge of mice with *P. brasiliensis*. Results are for daily doses of itraconazole. Horizontal bars show treatment days. Symbols: ●, no treatment; ○, polyethylene glycol (diluent for itraconazole); ■, 10 mg of itraconazole per kg; □, 50 mg of itraconazole per kg; ▲, 200 mg of itraconazole per kg. (From reference 184, with permission.)

Imidazole Derivatives

The introduction of imidazole derivatives in clinical practice improved the prognosis for and facilitated the therapy of paracoccidioidomycosis patients (246). At present, both ketoconazole and itraconazole have been studied thoroughly, while fluconazole and saperconazole await further observations concerning long-term remissions (246).

The first of the orally administered azoles, ketoconazole, was employed for paracoccidioidomycosis in 1978 and soon demonstrated its efficacy (215, 255). More than 90% of patients responded to the administration of 200 to 400 mg/day for 12 months or less. Most external lesions resolved within 3 to 6 months, and there was gradual clearing of lung lesions (215, 255), but fibrosis, which was unaffected by therapy, developed (132, 256). Approximately 5% of patients were nonresponders, and the relapse rate 3 years postherapy was lower (11%) than that with the older therapies (246, 277). Side effects were also minor and were primarily gastrointestinal or endocrine (gynecomastia, decreased libido) disturbances (215, 255). The only requirement for proper ketoconazole therapy is an acidic gastric pH. In patients with concurrent tuberculosis treated with rifampin (246), ketoconazole levels are drastically reduced and should be monitored.

In examining the costs of modern therapies, Pripas (234) calculated that the combined and prolonged use of amphotericin B plus sulfonamides is more expensive than a 6-month course of ketoconazole.

Because of the inhibition of peroxidase and catalase activities by various antimycotics, the erythrocyte metabolism of patients undergoing sulfadoxin or ketoconazole therapy was investigated. No changes were recorded in the former group, while patients on ketoconazole had decreased activity of antioxidant enzymes (glucose 6-phosphate dehydrogenase and glutathione reductase) (18). Consequently, patients with paracoccidioidomycosis who have erythrocyte enzyme defects should be monitored during treatment with ketoconazole (18).

The results of ketoconazole therapy appeared difficult to surpass, but a newer triazole derivative, itraconazole, soon demonstrated its superiority. Its higher activity, which means both a shorter period of therapy (6 months) and a lower dosage (100 mg/day), coupled with its lack of interference with endocrine metabolism make itraconazole the current drug of choice (212, 218). Even though the response rate and the promptness with which lesions heal are similar to those with ketoconazole (212), the advantages noted above tip the balance in favor of the newer triazole derivative. Furthermore, relapses occur in a lower proportion of cases (3 to 5%) (206, 218). To date, experience indicates that itraconazole is a safe drug even in patients with the severe

juvenile form of the mycosis (222). As with ketoconazole, itraconazole requires an acid pH to be properly absorbed and, consequently, antacids and beta blockers are contraindicated during therapy (218).

Other triazoles, among them fluconazole, are presently undergoing clinical trials. Fluconazole has been tried in some paracoccidioidomycosis patients (90); to date, the results indicate that this triazole is also highly active. One of its advantages is its solubility in water, which allows rapid penetration into the fluid compartments of the patient; this drug is also available for parenteral administration. The real efficacy of fluconazole will be proved by prolonged follow-up studies. For the newest of the triazoles, saperconazole, nine patients treated with an oral dose of 100 mg/day showed prompt responses, with improvement of symptoms and healing of mucocutaneous lesions in less than 2 months. X-ray alterations also improved early (3 to 6 months) (110).

These data indicate that there are now a number of options for effective therapy. Even a garlic extract has been shown capable of inhibiting the growth of *P. brasiliensis* (291). It must be recognized, however, that although the disease can be controlled with the antimycotics presently available, the problem of fibrotic sequelae, especially in the lungs, still constitutes a major obstacle to the patient's full recovery (55, 170, 212).

Table 9 summarizes the characteristics of the various therapies presently used for the treatment of paracoccidioidomycosis.

FUTURE PROSPECTS

Judging from the number of publications on paracoccidioidomycosis and its causative agent *P. brasiliensis* during the past 5 years (168 of 330 references), we can expect even greater progress in the next 5 years, thanks to new technologies and diagnostic reagents. In this era of advances in biochemistry, immunology, and genetics, we anticipate a greater understanding of immunoregulation during the disease and a deeper knowledge of the cell biology, i.e., genes and their products, of *P. brasiliensis*. These advances will permit a rational approach to specific immuno- or chemotherapy.

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